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Analysis of the Metallothionein Gene in Age-Related Macular Degeneration

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Purpose: To analyze the expression of metallothionein in neovascular membranes in patients with age-related macular degeneration (AMD) and to compare the findings in patients with proliferative diabetic retinopathy and proliferative vitreoretinopathy with findings in normal retinal pigment epithelial (RPE) cells.

Methods: Semiquantitative reverse transcriptase-polymerase chain reaction and immunohistochemical examinations were performed. Sequence analysis was also carried out.

Results: Expression of the metallothionein-II gene was not statistically significant in proliferative membranes. Sequence analysis revealed that there were at least six polymorphisms in the metallothionein-II gene and three in the metallothionein-IA gene. However, no amino acid substitution was observed. Metallothionein expression was also observed by immunohistochemical techniques in RPE cells of neovascular membranes in AMD patients.

Conclusions: Metallothionein was reported to be decreased in aged macular RPE and in some monkey retina with early onset of macular degeneration. However, our results suggest that the expression of the metallothionein-II gene in neovascular membranes in patients with AMD may be controlled like other proliferating cells. **Jpn J Ophthalmol 2000;44:115–121** © 2000 Japanese Ophthalmological Society

Key Words: Age-related macular degeneration, choroidal neovascular membrane, metallothionein, reverse transcriptase-polymerase chain reaction.

Introduction

Retinal pigment epithelial (RPE) cells play an important role in maintaining the microenvironment between the sensory retina and the choriocapillaris. Retinal pigment epithelial cells continuously engulf the outer segment of photoreceptor cells about 3×10^8 discs during a 70-year life span and, in part, play the scavenger under oxidative stress in the microenvironment.[1] These cells also have numerous other functions, such as blocking the influx of toxic substances into the retina or actively taking up nutrition as well as retinoid from the choriocapillaris.[2,3] Any dysfunction in these processes may lead to photoreceptor degeneration and to blindness. Royal College of Surgeon rats are a well-known animal model to demonstrate dysfunction of RPE cells.[4] In humans, genetically governed disease or damage by several acquired factors of the RPE[5] or underlying choriocapillaris[6] may generate photoreceptor death. One good model of human disease may be age-related macular degeneration (AMD).[7] In spite of the lesion being localized in the retina, the loss of vision is profound in affected patients. Allikmets and coworkers[8] have reported that AMD may be promoted by the mutation of the photoreceptor rod-specific ATP-binding transporter gene; however, the cause of AMD is still unknown.

Some authors have studied the RPE's function as a scavenger and a cause of AMD. Antioxidant enzyme activity has been examined by Newsome and coworkers.[9] Tate and colleagues[10] have reported an age-related decrease of metallothionein (MT) in human macular retinal pigment epithelium. Many types of MT are generated from a complex multigene family defined as low molecular weight, cysteine-rich, heavy metal binding proteins.[11] Although MTs do not show enzymatic activities, multifunc-

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tional roles in the body have been proposed, such as chelator to harmful heavy metals, scavenger to various radicals and active oxygen species, and regulator in the cell proliferation process. Suppression of MT synthesis was also reported in the retina of cynomolgus monkeys that showed early onset of macular degeneration.[12] Ishihara and coworkers[13] reported that patients with AMD manifest subnormal levels of serum zinc and vitamin E. Zinc and MT are also believed to protect cells from oxidative damage.[14–16] In this study, we examined the gene expression and the sequences of MT in subretinal neovascular membranes that were excised during vitreous surgery in patients with AMD, and compared them to those in other proliferative membranes in the eye and in normal RPE cells.

Materials and Methods

Ocular Tissues

Three-port vitrectomy was performed, with or without lensectomy and intraocular lens implantation, following excision of choroidal neovascular membranes from an artificial microhole of the retina in 11 patients with AMD (Table 1), aged between 44 and 77 years (average, 65.5 years). Sixteen samples from these 11 patients were examined by molecular biologic techniques. Five of the 16 samples were also used for immunohistologic study. For comparative study, 9 proliferative membranes from patients with proliferative diabetic retinopathy (PDR) and 7 from patients with proliferative vitreoretinopathy (PVR) were obtained by the same method described earlier.

Table 1. Cases of Age-Related Macular Degeneration

Case No.	Age	Sex	Laterality	Duration*	Visual Acuities Pre/Post Surgery
1	77	M	R	5	0.2/0.03
2	77	M	L	13	0.02/0.02
3	53	M	R	3	0.3/0.2
4	66	M	L	60	CF/0.03
5	65	M	L	10	0.03/0.03
6	44	M	L	5	0.01/0.5
7	76	M	R	46	0.02/0.04
8	66	F	R	4	0.2/0.02
9	56	M	L	2	HM/HM
10	69	M	L	25	0.03/0.1
11	65	M	R	31	0.1/0.1

*Duration indicates months between onset of symptoms and surgery. M: male, F: female, R: right, L: left, HM: hand motion, CF: counting fingers.

After vitreous surgery, removal of the subretinal neovascular membranes or preretinal proliferative membranes were observed by phase contrast microscopy (Olympus, PM-10ADS; Olympus, Tokyo).

Human RPE Cell Preparation

Human RPE cells were obtained, as previously reported.[17] In brief, after removing the anterior segment and vitreous, the eye cups were incubated with trypsin (0.05%)/ethylenediaminetetraacetic acid (0.53 mM) (Gibco BRL, Bethesda, MD, USA) solution in Hank's balanced salt solution (HBSS) (Gibco) for 30 minutes at 37°C in 5% CO_2. After separation of the neural retina, the eye cups were washed twice with HBSS. By performing pipetting under the dissecting microscope, we could collect RPE in 20% fetal bovine serum in F-12 medium (Gibco). Cell number was determined with the use of a hemocytometer.

Extraction of mRNA, cDNA Generation and Reverse Transcriptase-Polymerase Chain Reaction

mRNAs were extracted from these materials by using oligo dT cellulose (Pharmacia Biotech, Uppsala, Sweden).[17] Briefly, the cells were pelleted and suspended in extraction buffer (4 M guanidinium thiocyanate and 0.5% *N*-lauroyl sarcosine); the cellular homogenate was cleared by centrifugation and mixed with oligo dT cellulose. The oligo dT cellulose was washed with high salt buffer (10 mM Tris–HCl [pH 7.5], 1 mM EDTA, 0.5 M NaCl) several times, followed by low salt buffer (10 mM Tris–HCl [pH 7.5], 1 mM EDTA, 0.1 M NaCl) several times. Then mRNA was eluted by prewarmed elution buffer (10 mM Tris–HCl [pH 7.5], 1 mM EDTA). First-strand cDNA was generated by random hexadeoxynucleotides at 0.2 μg in each reaction, which was catalyzed by Moloney Murine Leukemia Virus reverse transcriptase (Pharmacia Biotech).

With the use of a thermocycler (Perkin Elmer, Norwalk, CT, USA), polymerase chain reaction (PCR) was carried out in 50 μL of reaction mixture; 20 μM of each primer; 200 μM each of dATP, dCTP, dGTP, and dTTP; 50 mM of KCl; 10 mM of Tris–HCl (pH 8.3); 1.5 mM of $MgCl_2$ and 0.001% gelatin; and 2.5 units of *Taq* polymerase. Reaction cycles were 35. The temperature settings for PCR were 94°C for 1 minute for denaturation, 60°C for 2 minutes for annealing, and 72°C for 2 minutes for polymeration. In each case, amplified DNA was separated in 1.5% agarose gel (SeaKem; FMC BioProducts, Rock-

land, ME, USA) containing 0.05 mg/mL ethidium bromide. DNA was visualized using an ultraviolet transilluminator.

Primer Preparations

The primer sets for the MT-II gene[18] amplified 258 base pair (bp), MT-IA 314 bp,[19] and β-actin 313 bp[17] were as follows: For MT-II: (sense probe) 5′-GACTCTAGCCGCCTCTTCAG-3′ and (anti-sense probe) 5′-TTGTGGAAGTCGCGTTCT-3′. For MT-IA: (sense probe) 5′-CTCTCAACTTCTTGCTTGGG-3′ and (anti-sense probe) 5′-AATGGGTCAGGGTTGTATGG-3′. For β-actin: (sense probe) 5′-CTACAATGAGCTGCGTGTGG-3′ and (anti-sense probe) 5′-CGGTGAGGATCTTCATGAGG-3′

Semiquantitative PCR

Semiquantitative PCR was performed by the modification methods of Moore and coworkers.[20] Briefly, PCR was performed under the conditions described, with variable cycles and concentrations of cDNA. The PCR products were separated on 1.5% agarose gel and photographed for further analysis; the photographs were quantitatively analyzed by the NIH image analyzer on the computer screen. Under the exponential phase of the PCR products, the quantity of the PCR products were compared. β-actin was used as an internal control.

Sequence Analysis

The amplified PCR products were subsequently subcloned into a T-vector (Promega, Madison, WI, USA). Subcloned PCR products were then sequenced with an automatic DNA sequencer (Pharmacia LKB ALF DNA sequencer; Pharmacia, Uppsala, Sweden) using a dideoxy chain termination protocol.[21]

Immunocytochemical Analysis

Immunohistochemical study was performed according to the modified method of Kusakari and coworkers.[22] Briefly, materials were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) or 10% formalin. The fixed tissues were embedded in paraffin. The sections were incubated overnight at 4°C with anti-MT antibody, which was confirmed to be immunoreactive to both MT-I and MT-II (Zymed Laboratory, San Francisco, CA, USA) or anti-cytokeratin antibody (a monoclonal antibody mixture; Sigma, St. Louis, MO, USA), and then with 1:400 diluted anti-mouse IgG-gold conjugate (10 nm gold particles, OD525 = 0.6; British BioCell International, Cardiff, UK) 2 hours at room temperature. Antibodies were dissolved in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 1% FBS, and sections were washed 3 times for 5 minutes with PBS containing 0.05% Tween 20 in between incubations. Silver deposit was developed by a silver enhancing kit (British BioCell) for 10–20 minutes at room temperature. The sections were dehydrated and mounted. The prepared sections were then stained with hematoxylin and eosin. We also performed this immunocytochemical investigation using serial sections.

Statistical Analysis

Statistical analysis was done using a Kruskal-Wallis or Pearson correlation coefficient test. *P* values less than .05 were judged to be statistically significant.

Human Study

The tenets of the Declaration of Helsinki were followed, and informed consent was obtained from all subjects who participated in this study.

Results

We performed three-port vitreous surgery to remove the subretinal neovascular membranes from patients with AMD. Neovascular membranes in patients with AMD and proliferative membranes in patients with PDR or PVR were obtained successfully without major complications. The average age in patients with AMD was 65.5 years; PDR, 53.3 years; and PVR, 56 years. A total of 16 neovascular proliferative membranes were obtained from the 11 patients with AMD. A total of 9 proliferative membranes from the patients with PDR and 7 proliferative membranes from the patients with PVR were used for the studies described later. The duration between onset and removal of submacular neovascular membranes varied among patients (Table 1). Visual acuities before and after surgery also differed. We monitored the subretinal membranes in patients with AMD before and after surgery by scanning laser ophthalmoscopy. After vitreous surgery, removal of the subretinal neovascular membranes was observed by phase contrast microscopy. We always confirmed that the membranes included cells containing pigment (Figure 1).

Human RPE cells were obtained from the 11 individuals, as described in Materials and Methods. As we reported previously,[13] the expressions of cellular retinaldehyde-binding protein and tyrosinase-related

Jpn J Ophthalmol
Vol 44: 115–121, 2000

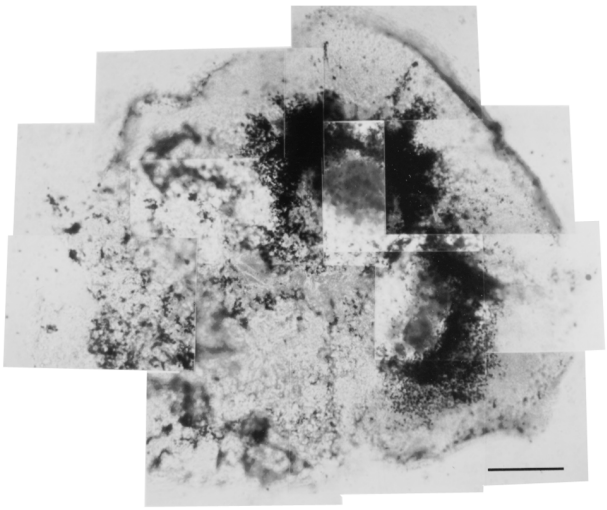

Figure 1. Subretinal neovascular membranes excised during vitreous surgery in patients with age-related macular degeneration (case 3, Table 1). Bar = 200 μm.

protein-1 genes were observed in these cells. These gene expressions were also confirmed in the proliferative membranes of the patients with AMD, PVR, and PDR (data not shown).

From the constant results of β-actin, reverse transcriptase-polymerase chain reaction (RT-PCR) was thought to be performed successfully. Reverse transcriptase-polymerase chain reaction showed that the MT-II gene was amplified in all the samples examined (Figure 2A). Conversely, negative or ± gene amplification was observed in the MT-IA gene (Figure 2B). The RT-PCR of β-actin from the same samples are shown (Figure 2C). The PCR products were always in the exponential phase under the various PCR cycles, and they appeared to be linear under the experimental mRNA concentrations. Semiquantitative RT-PCR and statistical analysis showed that the expression of the MT-II gene in the samples from AMD patients was not decreased if we compared the results with those from PVR patients (P = .2367), PDR (P = .2396), and normal RPE (P = .9232) (data not shown). These results did not appear to differ from each other. Statistical analysis also revealed that no correlation was observed between the duration of the disease and the expression of the MT-II gene (Pearson correlation coefficient test). The expressions of MT-IA gene were so weak that we did not perform statistical analysis.

After subcloning into the T-vector of the PCR products, sequence analyses of MT-II (cases 1, 2, 3, and 5) and MT-IA (cases 4, 6, and 8) genes were performed in cases 1, 2, and 5 (Figure 3A and 3B). We

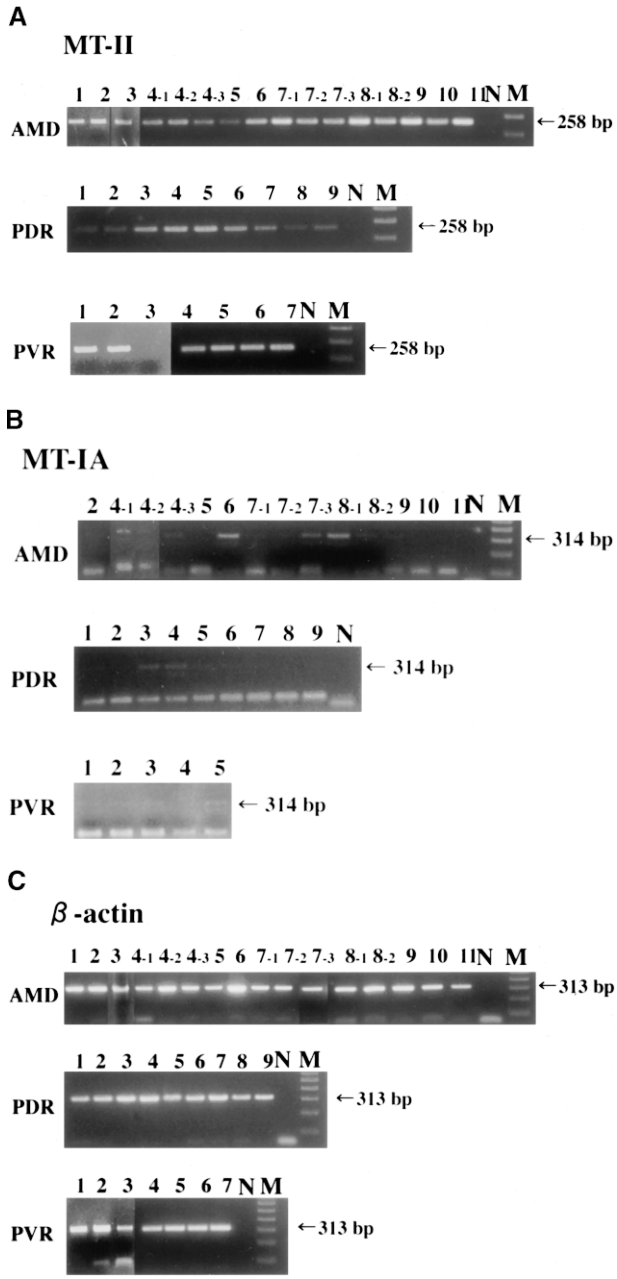

Figure 2. Results of reverse transcriptase-polymerase chain reaction (RT-PCR) of (**A**) metallothionein II and (**B**) metallothionein-IA. We confirmed that PCR products were always in the exponential phase during various PCR cycles, and that they appeared linear under experimental mRNA concentrations. Semiquantitative analysis of the gene was performed using internal control of β-actin, as shown in (**C**). N indicates negative control, M; 100 base pair ladder.

could find no nucleotide substitutions, which substitute amino acid sequences, in either the MT-II or -IA genes. However, there were at least 6 polymorphisms in the sequences of the MT-II gene and 3 in the MT-IA

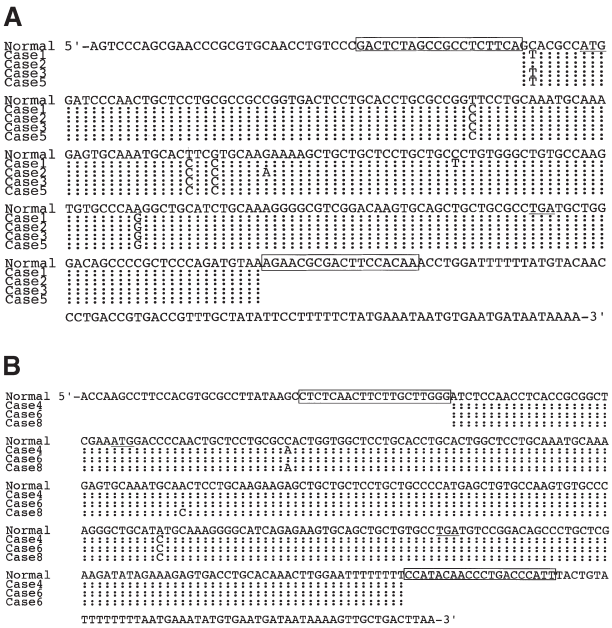

Figure 3. Results of sequence analyses of (**A**) metallothionein-II (MT-II) and (**B**) metallothionein-IA (MT-IA) genes, which were transcribed in the neovascular membranes in patients with age-related macular degeneration, as shown. Neovascular membranes used for sequence analysis were from cases 1, 2, 3, and 5 in MT-II and cases 4, 6, and 8 in MT-IA (Table 1). Boxes indicate primers for amplifying each DNA. Normal indicates sequence that has already been reported.

gene in our Japanese patients, if we compare our results with those previously reported.[14,15] The sequence analysis was repeated from both DNA strands using different clones.

From the results of the cytokeratin immunohistochemical study, the cells containing pigment in the excised membranes from the subretinal spaces in AMD patients were thought to be RPE cells (Figure 4). Strong immunostaining was also observed with anti-MT antibody at almost the same place as with cytokeratin antibody (Figure 4). These results showed a strong expression of MT in the RPE of proliferative membranes in AMD patients.

Discussion

Metallothioneins have been thought to play an important role in the metabolism of some metals such as zinc, copper, and cadmium by binding and releasing them.[23] It also has been believed that MTs act as a scavenger of active oxygen stress[12,14] and protect cells from peroxidation[15] and from radiation-induced hydroxyl radicals.[16] Metallothioneins are widely distributed and expressed in actively proliferating and differentiating tissues, and have shown weak expression in relatively long turnover cells,[24] which may be introduced from the responsible promoter sequences.[19] MTs are also induced by various metals, hormones, cytokines, and endogenous and exogenous agents.[14,25] They derive from a complex multigene family that is clustered on chromosome 16 with several pseudogenes.[26] Some of the genes in this cluster have been characterized.[27,28] Nicolas and coworkers[12] reported the decreased expression of MT in the retina of a cynomologus monkey that showed early onset macular degeneration. In this study, we also examined the expression of two MT isoforms (MT-II and MT-IA) by semiquantitative RT-PCR in the proliferative membranes in patients with AMD, PDR, PVR, and also in normal RPE cells, using immunohistochemical techniques.

Cruickshanks and coworkers[29] reported that late-stage AMD appeared to be rare among the Hispanic population. Black races also reportedly have had relatively low rates of AMD.[30–32] Because the prevalence of AMD has been reportedly different among races, these authors have suggested that more heavily pigmented people may be at a lower risk for late-stage disease because of the protective effects of melanin against oxidative stress.[33,34] Some differences among races may be important factors for the generation of AMD. Although we could not find any statistical significance, we found at least 6 polymorphisms in the coding region of the MT-II gene and 3 in the MT-IA gene in our Japanese patients, when we compared the sequences with previously reported ones.[18,19] However, no amino acid substitution was observed in these genes in Japanese patients.

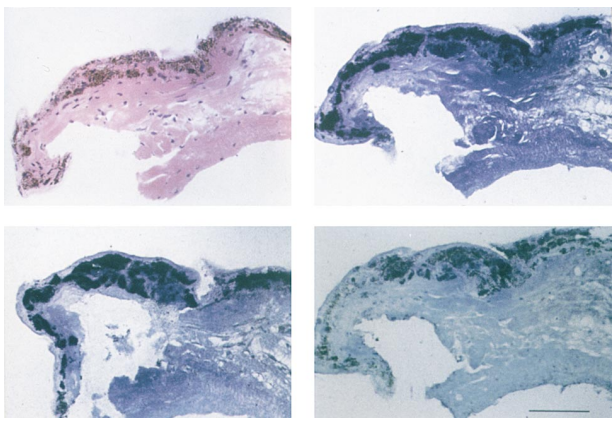

Figure 4. Results of immunohistochemical analyses of cytokeratin and metallothionein (MT). Prepared sections were stained with hematoxylin and eosin (top left), anti-cytokeratin antibody (top right), anti-MT antibody (bottom left), and without anti-MT (bottom right). Bar = 50 μm.

Jpn J Ophthalmol
Vol 44: 115–121, 2000

Nishimura and coworkers[24] reported that strong MT staining was observed in the epithelium of the lens, cornea, and retinal pigment epithelium of rats. We also demonstrated MT expression in the RPE of neovascular membranes from AMD patients by immunohistochemical examination. Based on histological examination, Nasir and coworkers[7] reported that all excised neovascular membranes from patients included RPE cells. In pathological conditions, such as AMD or other intraocular proliferative diseases, RPE cells may proliferate, and the expression of MT may be further induced. The expression of MTs in RPE cells may be coincident with the findings that retina and placenta act as a barrier for preventing the influx of toxic substances, such as heavy metal ions, into the retina.[24]

Tate and coworkers[10] reported the age-related decrease of MT in the RPE of the macula. We examined the proliferative membranes (not of aged RPE cells), which may include not only RPE cells but other cell types. Differences in cell constitution may affect the results. Further examination, such as in situ hybridization results compared with findings in age-related RPE in the macula, may be necessary for detecting the responsible cells. However, from the results of semiquantitative RT-PCR using neovascular membranes from patients with AMD, we found no statistical significance in the expression of the MT-II gene compared with findings in PVR, PDR, and normal RPE cells. Our results suggest that the expression of the MT-II gene in neovascular membranes in AMD patients may be controlled like other proliferating cells, such as glial cells or other cell types reported in other tissues.[35]

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References

1. Marshall J. The ageing retina: physiology or pathology. Eye 1987;1:282–95.
2. Berman ER. Retinal pigment epithelium. In: Berman ER, ed. Biochemistry of the eye. New York: Plenum Press, 1991:380–406.
3. Bok D. Retinal photoreceptor-pigment epithelium interactions. Friedenwald lecture. Invest Ophthalmol Vis Sci 1985;26:1659–94.
4. Bok D, Hall MO. The role of the pigment epithelium in the etiology of inherited retinal dystrophy in the rat. J Cell Biol 1971;49:664–82.
5. Sarks SH. Ageing and degeneration in the macular region: a clinico-pathological study. Br J Ophthalmol 1976;60:324–41.
6. Friedman E. A hemodynamic model of the pathogenesis of age-related macular degeneration [editorial]. Am J Ophthalmol 1997;124:677–82.
7. Nasir MA, Sugino I, Zarbin MA. Decreased choriocapillaris perfusion following surgical excision of choroidal neovascular membranes in age-related macular degeneration. Br J Ophthalmol 1997;81:481–9.
8. Allikmets R, Shroyer NF, Singh N, et al. Mutation of the Stargardt disease gene (ABCR) in age-related macular degeneration. Science 1997;277:1805–7.
9. Newsome DA, Swartz M, Leone NC, Elston RC, Miller E. Oral zinc in macular degeneration. Arch Ophthalmol 1988;106:192–8.
10. Tate DJ Jr, Newsome DA, Oliver PD. Metallothionein shows an age-related decrease in human macular retinal pigment epithelium. Invest Ophthalmol Vis Sci 1993;34:2348–51.
11. Kagi JH, Schaffer A. Biochemistry of metallothionein. Biochemistry 1988;27:8509–15.
12. Nicolas MG, Fujiki K, Murayama K, et al. Studies on the mechanism of early onset macular degeneration in cynomolgus monkeys. II. Suppression of metallothionein synthesis in the retina in oxidative stress. Exp Eye Res 1996;62:399–408.
13. Ishihara N, Yuzawa M, Tamakoshi A. Antioxidants and angiogenetic factor associated with age-related macular degeneration (exudative type). Nippon Ganka Gakkai Zasshi (J Jpn Ophthalmol Soc) 1997;101:248–51.
14. Sato M, Sasaki M, Hojo H. Antioxidative roles of metallothionein and manganese superoxide dismutase induced by tumor necrosis factor-alpha and interleukin-6. Arch Biochem Biophys 1995;316:738–44.
15. Thomas JP, Bachowski GJ, Girotti AW. Inhibition of cell membrane lipid peroxidation by cadmium- and zinc-metallothioneins. Biochim Biophys Acta 1986;884:448–61.
16. Thornalley PJ, Vasak M. Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. Biochim Biophy Acta 1985;827:36–44.
17. Abe T, Durlu YK, Tamai M. The properties of retinal pigment epithelial cells in proliferative vitreoretinopathy compared with cultured retinal pigment epithelial cells. Exp Eye Res 1996;63:201–10.
18. Karin M, Richards RI. Human metallothionein genes: molecular cloning and sequence analysis of the mRNA. Nucleic Acids Res 1982;10:3165–73.
19. Richards RI, Heguy A, Karin M. Structural and functional analysis of the human metallothionein-IA gene: differential induction by metal ions and glucocorticoids. Cell 1984;37:263–72.
20. Moore NC, Anderson G, Williams GT, Owen JJ, Jenkinson EJ. Developmental regulation of bcl-2 expression in the thymus. Immunology 1994;81:115–9.
21. Sanger F, Coulson AR, Barrell BG, Smith AJ, Roe BA. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J Mol Biol 1980;143:161–78.
22. Kusakari Y, Nishikawa S, Ishiguro S, Tamai M. Histidine-like immunoreactivity in the rat retina. Curr Eye Res 1997;16:600–4.
23. Webb M, Cain K. Functions of metallothionein [review]. Biochem Pharmacol 1982;31:137–42.
24. Nishimura H, Nishimura N, Kobayashi S, Tohyama C. Immunohistochemical localization of metallothionein in the eye of rats. Histochemistry 1991;95:535–9.
25. Bauman JW, Liu YP, Andrews GK, Klaassen CD. Examination of potential mechanism(s) of metallothionein induction by diethyl maleate. Toxicol Appl Pharmacol 1992;117:226–32.
26. Schmidt CJ, Hamer DH. Cloning and sequence analysis of two monkey metallothionein cDNAs. Gene 1983;24:137–46.
27. Palmiter RD, Findley SD, Whitmore TE, Durnam DM. MT-

III, a brain-specific member of the metallothionein gene family. Proc Natl Acad Sci 1992;89:6333–7.

28. Stennard FA, Holloway AF, Hamilton J, West AK. Characterisation of six additional human metallothionein genes. Biochim Biophys Acta 1994;1218:357–65.
29. Cruickshanks KJ, Hamman RF, Klein R, Nondahl DM, Shetterly SM. The prevalence of age-related maculopathy by geographic region and ethnicity. The Colorado-Wisconsin Study of Age-Related Maculopathy. Arch Ophthalmol 1997;115: 242–50.
30. Gregor Z, Joffe L. Senile macular changes in the black African. Br J Ophthalmol 1978;62:547–50.
31. Klein R, Rowland ML, Harris MI. Racial/ethnic differences in age-related maculopathy. Third National Health and Nutrition Examination Survey [published erratum appears in Ophthalmology 1995;102(8):1126]. Ophthalmology 1995;102: 371–81.
32. Schachat AP, Hyman L, Leske MC, Connell AM, Wu SY. Features of age-related macular degeneration in a black population. The Barbados Eye Study Group. Arch Ophthalmol 1995;113:728–35.
33. Jampol LM, Tielsch J. Race, macular degeneration, and the Macular Photocoagulation Study. Arch Ophthalmol 1992;110: 699–700.
34. Pieramici DJ, Bressler NM, Bressler SB, Schachat AP. Choroidal neovascularization in black patients. Arch Ophthalmol 1994;112:1043–6.
35. Nishimura H, Nishimura N, Tohyama C. Immunohistochemical localization of metallothionein in developing rat tissues. J Histochem Cytochem 1989;37:715–22.