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ScienceDirect
Experimental Eye Research 86 (2008) 856–859
EXPERIMENTAL EYE RESEARCH
www.elsevier.com/locate/yexer

Letter to the Editor

Immunohistochemical analysis of aldehyde-modified proteins in drusen in cynomolgus monkeys (*Macaca fascicularis*)☆

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Received 20 December 2007; accepted in revised form 14 January 2008
Available online 31 January 2008

Abstract

Protein modifications resulting from reactive aldehydes are thought to be involved in the pathogenesis of various degenerative diseases. Aged cynomolgus monkey (*Macaca fascicularis*) spontaneously develop drusen in the macula, consistent with the phenotype observed in early-stage age-related macular degeneration (AMD), indicating that this animal is an optimum model for AMD. In retinal sections from three monkeys with macular degeneration, regardless of their size, drusen were consistently positive with immunohistochemical labeling against protein modifications by 4-hydroxynonenal and 4-hydroxyhexenal, end products of non-enzymatic oxidation of n-6 and n-3 polyunsaturated fatty acids, respectively. Positive labeling for both modifications was observed in the ganglion cell layer, the inner nuclear layer, the outer nuclear layer, and the retinal pigment epithelium. However, no consistent differences in location or intensity of the labeling were observed between monkeys with normal macula and macular degeneration. The results suggest a possible association between drusen formation and protein modifications by aldehydes in the pathogenesis of AMD.
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Keywords: aldehydes; 4-hydroxynonenal; 4-hydroxyhexenal; cynomolgus monkey; drusen; age-related macular degeneration; protein modification

Age-related macular degeneration (AMD) is the leading cause of legal blindness in elderly individuals in industrialized countries (Fine et al., 2000). Accumulation of extracellular deposits between the retinal pigment epithelium (RPE) and Bruch's membrane, referred to as drusen, is regarded as a hallmark risk factor for development of AMD (de Jong, 2006). Various lipids, polysaccharides, and glycosaminoglycans have been identified as constituents of drusen (Hageman et al., 2001). Recent studies have revealed that drusen contains various protein molecules that are related to inflammation, immune responses, and oxidative stresses (Mullins et al., 2000; Crabb et al., 2002); yet the mechanism of formation is not fully understood.

Aged monkeys spontaneously develop macular degenerative changes such as pigment mottling, hyperpigmentation or hypopigmentation, and drusen in the macula, consistent with the phenotype observed in early-stage AMD (Stafford et al., 1984; Ishibashi et al., 1986). Previous proteomic analysis indicated that a number of protein components are common in drusen from monkeys and humans (Crabb et al., 2002; Umeda et al., 2005). Thus, these animals are thought to be an optimum animal model for AMD.

4-Hydroxynonenal (4-HNE) and 4-hydroxyhexenal (4-HHE) are α,β-unsaturated aldehydes that are end products of non-enzymatic oxidation of n-6 and n-3 polyunsaturated fatty acids, respectively (Esterbauer, 1993). These highly reactive aldehydes can react readily with histidine, cysteine, or lysine

☆ The authors have no proprietary interest in any aspect of this report.

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0014-4835/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.exer.2008.01.016

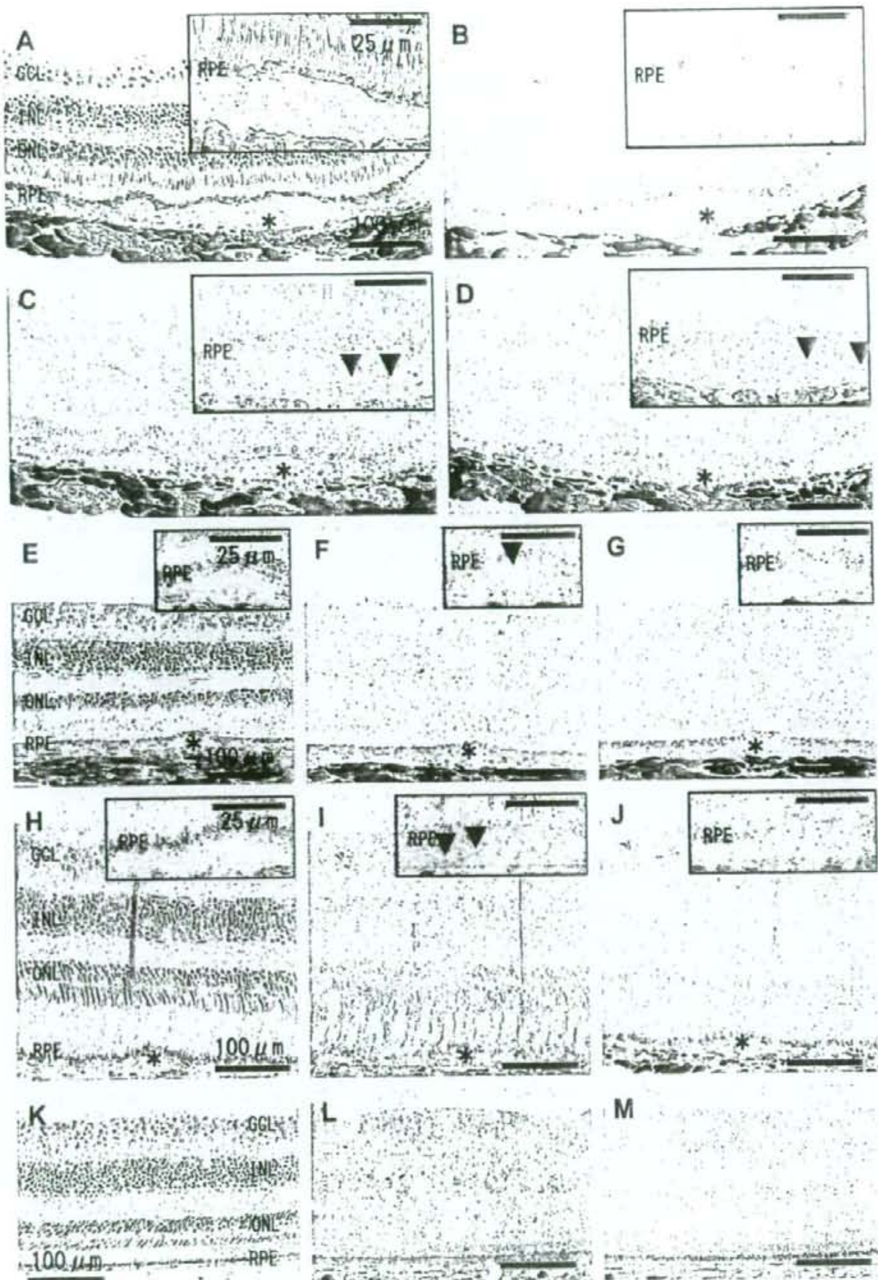

Fig. 1. Retinal sections from monkeys with macular degeneration and normal macula. Representative retinal sections from three monkeys with macular degeneration (panels A–D, E–G, and H–J, respectively) and those from a monkey with a normal macula (panels K–M) are shown. Representative sections stained with hematoxylin and eosin (panels A, E, H, and K) labeled with normal mouse IgG (panel B), 4-HNE-modified proteins (panels C, F, I, and L) and 4-HHE-modified proteins (panels D, G, J, and M). Asterisks and arrowheads indicate drusen and granular labeling, respectively. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; and RPE, retinal pigment epithelium.

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residues of proteins, leading to formation of stable Michael adducts with a hemiacetal structure (Uchida and Stadtman, 1992). Formation of these adducts leads to a variety of cytopathological effects such as inhibition of enzyme activity, inhibition of protein, RNA, and DNA synthesis, cell cycle arrest, and apoptosis (Awasthi et al., 2004). The use of specific antibodies to recognize the hemiacetal structure of Michael adducts enables their detection in tissues (Uchida et al., 1993). Previous studies have suggested that modifications by these aldehydes of proteins common to drusen components in humans are molecular events preceding light-induced retinal degeneration in rats (Tanito et al., 2005, 2006). Recently, proteins modified by 4-HNE were detected in the neural retina of patients with AMD (Ethen et al., 2007). We evaluated immunohistochemically the localization of proteins modified by these aldehydes on retinal sections from aged monkeys.

Cynomolgus monkeys (*Macaca fascicularis*) were reared in large-scale breeding facilities at the Simian Conservation Breeding and Research Center, Inc., Manila, the Philippines. Maintenance of monkeys and preparation of paraffin-embedded retinal sections have been described previously (Umeda et al., 2005). At the breeding facilities, 278 aged female monkeys (age range, 13–25 years; average age, 16.9 years) were funduscopically examined three times from 2001 to 2004. Of the 278 animals, 67.6% had a normal macula with no detectable pigmentary abnormalities, 10.8% were diagnosed with mild macular degeneration (<5 drusen), 11.2% with moderate macular degeneration (5–20 drusen), and 10.4% with severe macular degeneration (>20 drusen) (Umeda et al., 2005).

In the current study, retinal sections from three monkeys with normal macula and three monkeys with severe macular degeneration were examined. The immunohistochemistry methods were described previously (Tanito et al., 2005). Mouse anti-4-HNE- and anti-4-HHE-modified protein antibodies were purchased from NOF Corporation (Tokyo, Japan). Hematoxylin and eosin staining was performed to observe accumulation of drusen of various sizes between the RPE and choriocapillaries in the macular region in retinal sections from the three monkeys with severe disease (Fig. 1A, E, and H), whereas no drusen accumulated in any retinal sections from three monkeys with normal macula (Fig. 1K). By immunohistochemistry, regardless of size, drusen were consistently positive with diffuse labeling against both protein modifications by 4-HNE (Fig. 1C, F, and I) and 4-HHE (Fig. 1D, G, and J) in retinal sections from three monkeys with macular degeneration. Using high magnification, drusen frequently contained granular labeling against both protein modifications by 4-HNE (insets, Fig. 1C, F, and I) and 4-HHE (inset, Fig. 1D). Positive labeling for both antibodies was observed in the ganglion cell layer, the inner nuclear layer, the outer nuclear layer, and the RPE. However, no consistent differences in localization or intensity of labeling were observed between monkeys with normal macula and those with macular degeneration. Retinal sections incubated with normal mouse IgG as a negative control showed no positive staining over all retinal layers. The results clearly showed that the drusen contained proteins modified by the reactive aldehydes, 4-HNE and 4-HHE.

AMD progression can be slowed with antioxidant and zinc (The AREDS Research Group, 2001), thus oxidative stress has been suspected of contributing to the pathogenesis of AMD (Beatty et al., 2000). The relationship between abnormal protein oxidation/modification and macular degeneration has been reported, including detection of cross-linked species of tissue metalloproteinase inhibitor 3 and vitronectin, docosahexaenoic acid-derived carboxyethylpyrrole protein adducts in drusen from patients with AMD (Crabb et al., 2002), and protein modifications from oxidized carbohydrate such as carboxymethyl-lysine and pentosidine in ocular tissues from aged donors and those with AMD (Ishibashi et al., 1998; Handa et al., 1999). In addition to the previous evidence, we identified possible involvement of protein modifications by 4-HNE and 4-HHE in the formation of drusen in AMD. Protein modification by biologically active molecules including reactive aldehydes may be a critical process in drusen formation and the development of AMD.

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Comparative Proteomic Analyses of Macular versus Peripheral Retina in Cynomolgus Monkeys (*Macaca fascicularis*)

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Keywords

Macula, Retina, Age-related macular degeneration, Tropomyosin, Synuclein,

Ribonucleoprotein, Mn-superoxide dismutase, Photoreceptor

Abstract

Macula is the specialized region of the retina responsible for high resolution visual acuity in primates. To investigate regional differences between macula and peripheral retina, proteomic analyses of tissues from cynomolgus monkeys were compared. By two dimensional gel electrophoresis and mass spectrometry, twenty six proteins were identified from spots detected only in the macular tissues. These proteins may play a role in the pathogenesis and progression of macular disease.

Introduction

The central region of the retina is called the macula and is approximately 1.5 to 2.0 mm in diameter in humans (1) and 0.6 to 0.9 mm in cynomolgus monkeys (2, 3). The fovea is located at the center of the macula approximately 0.35 mm in diameter where no blood vessels are formed. The density of cone photoreceptors in retina is highest in the fovea (4), and they are connected with large number of retinal ganglion cells (RGCs) in this small region (1). Any damage to the macula can have devastating effect on the central visual acuity e.g., in patients with macular dystrophies and age-related macular degeneration (AMD).

AMD is a multifactorial disease in which multiple genes and environmental factors are involved to progress mainly to two types of diseases (5). The wet-form of AMD is characterized by the loss of central vision caused by choroidal neovascularization (CNV) in subretinal space, while the dry-form of AMD is characterized as geographic atrophy of the retinal pigment epithelium (RPE) cells (1). The deposit called the "drusen" accumulates between the RPE and Bruch's membrane and is considered as hallmark of dry-form of AMD. Although previous reports have shown drusen as risk factor for AMD, explanation of its preferential accumulation in the macula still remains undetermined.

The uniqueness of the macular area is also demonstrated by the preferential development of CNV in the wet-form of AMD. These findings indicate that the macular region of the retina is different from the peripheral retina not only by morphology but also by protein distribution. It can be interpreted as difference of macula versus peripheral retina. Based on this hypothesis, comprehensive gene expression studies of the macula in comparison with peripheral retina using DNA microarray or serial analysis of gene expression (SAGE) have been performed (6-10). Sharon et al. have reported several genes preferentially expressed in the human macula and RPE by SAGE. Most of these genes were associated with the function of the RGC, and presumably detected because of the high density of RGC in the macular area (6). Rickman et al. also performed SAGE

on human retinas and isolated RPE cells and identified genes that were abundantly expressed in cones, RGC, and RPE cells (7). Ishibashi et al. performed 4 K DNA microarray analysis on RPE cells in the macula reporting five differentially expressed genes which was confirmed by real time PCR (8). Recently, Radeke et al. (9) and van Soest et al. (10) used 22 K DNA microarray and identified number of genes that were differentially expressed in the macula and peripheral retina. Few of these genes were highly expressed in the RPE cells in the macula. van Soest et al. showed that WAP four-disulfide core domain 1 was one of the highly expressed proteins in the RPE cells in the macular area by immunohistochemistry (10). However in many cases, the expression level of mRNA did not correlate with the expression level of the protein demonstrating the limitation of mRNA analyses for translational and posttranslational evaluations.

Recent technical advances in proteomics have allowed direct determination of the protein profile of body fluids and tissue homogenates. Proteomic analyses of the retina were first performed by Nishizawa et al. (11), and soon several groups catalogued the retinal proteins using single or two-dimensional (2D) gel electrophoresis followed by mass spectrometry analysis (12-14). Ethen et al. examined cadaver eyes with AMD by proteomic analyses and reported that the expression of proteins change with the progression of AMD, and the changes in the macula was different from that in the

peripheral retina (15).

Proteomic studies of the macula are difficult because of the lack of fresh human eyes, and the small area of the macula. To overcome these problems, we selected non-human primate eyes of the Cynomolgus monkey (*Macaca fascicularis*), which have a well defined macula. Monkeys with characteristics of AMD have been reported by many investigators (16-21) including our laboratory on early-onset and late-onset Cynomolgus monkey AMD models (22-26). Our proteomic study has shown that the protein profile of monkey eyes with drusen were similar to that in human drusen for many of the key molecules including complement components C5 and C9. In addition, the terminal C5b-9 complement complex was confirmed to present in drusen by immunostaining (27).

To identify the proteins present at high levels in the macular area and to understand the biology of the macula, we performed proteomic analyses to determine the protein profile of the macula and peripheral retina and posttranslational modification specific to the macular area.

Experimental section

Preparation of Cynomolgus monkey eyes

All primate experiments were approved by the Animal Ethics Committee of the Tsukuba Primate Research Center and were conducted in accordance with the The Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Eight eyes from eight normal female Cynomolgus monkeys (*Macaca fascicularis*) whose ages ranged between 13- to 19 years were studied. Eyes were removed approximately one hour after death and treated with RNAlater (Applied Biosystems, Tokyo, Japan). Then a 3 mm diameter macular and peripheral retina containing the RPE and choroidal layer were punched out and frozen until use. The proteins extracted from the tissues of eight eyes were pooled for analyses.

Protein extraction and 2D-gel electrophoresis

The proteins from the macula and peripheral retina were extracted after homogenization and sonication in sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 40 mM Tris, 0.2% Bio-Lyte 3/10 (Bio-Rad Hercules, CA)). After centrifugation for 15 min x 3 at 14,000 rpm (20,800 x g), the lysate was precipitated using Ready Prep 2D cleanup kit (Bio-Rad) and redissolved in sample buffer. The supernatant was collected. The protein concentrations were determined with the RC-DC protein assay kit (Bio-Rad) according to the manufacturer's instruction. Protein samples (300 μg) were separated by IEF using 17

cm IPG strips. After 12 to 16 hours of rehydration at 20° C, IEF was used for the first dimension with an initial voltage of 250 V for 15 min and then increased to 10,000 V for 3 hours and held until 60,000 volt-hours was reached. Immediately after the IEF, the IPG strips were stored at -20º C until the equilibration step was carried out. The IPG strips were equilibrated for 20 min in buffer containing 6 M urea, 2% SDS, 0.375 M Tris (pH 8.8), and 20% glycerol under reduced conditions with 2% DTT, followed by another incubation for 10 min in the same buffer under alkylating conditions with 2.5% iodoacetamide. Equilibrated IPG strips were electrophoresed for the second-dimension using 12% acrylamide gel. 2D-gel electrophoresis was performed at four different pH ranges, viz., pH 3-10, pH 4-7, pH 5-8 and pH7-10. After 2D-gel electrophoresis, the proteins were stained with SYPRO Ruby (Bio-Rad). The images of the macular and peripheral retina were compared with ImageMaster 2D Platinum ver.5.0 (GE Healthcare Bio-Sciences, Piscataway NJ) followed by a visual inspection. Then, the gel spots, as shown in Figure 1-4 (arrows) were excised. Butt et al. have described the difficulties of IEF separation by RNAlater because of contaminations (28). Our samples were desalted prior to the IEF separation by Ready Prep 2D cleanup kit (Bio-Rad) using TCA acetone precipitation which resulted in successful separations.

In gel digestion and LC-MS/MS analyses

Each gel piece was cut to approximately one cubic millimeter and washed twice with 50 mM ammonium bicarbonate/50% acetonitrile. After destaining, the gel pieces were rinsed with distilled water, and incubated with acetonitrile for 20 min. The supernatant was discarded and the gel pieces were completely dried before incubation with 10 mM DTT in 100 mM ammonium bicarbonate for 45 min at 56° C. The supernatant was discarded and the pieces were incubated in the dark with 55 mM iodoacetamide in 100 mM ammonium bicarbonate (30 min, at room temperature). The supernatant was discarded, and the gels were washed three times. Finally, the gel pieces were completely dried before tryptic digestion in a solution of sequencing grade modified trypsin (12.5 ng/ul; Promega, Madison, WI) in 50 mM ammonium bicarbonate. The digestion was performed at 37° C overnight, and the extraction step was performed once with 25 mM ammonium bicarbonate, twice with 5% formic acid, and finally with distilled water. The extracted peptides were pooled and dried. After re-suspending in 40 μl of aqueous 0.01% trifluoroacetic acid/2% acetonitrile, the samples were analyzed by LC-MS/MS.

LC-MS/MS was performed with a combined system of Paradigm system (Michrom BioResources, Auburn, CA) and an ESI mass spectrometer (LCQ Deca XP plus or Finnigan LTQ; Thermo Fisher Scientific, Yokohama, Japan; assembled by AMR Inc.,

Tokyo, Japan). For the LCQ analysis, sample peptides were separated on nano column (AMR Inc.) with solvent A (2% acetonitrile/0.1% formic acid) and B (90% acetonitrile/0.1 % formic acid) at a flow rate of 0.6 μl/min, gradient of 5 to 95% solvent B over 40 min. For the LTQ analysis, peptides were separated on Magic C18 (Michrom BioResources) with solvent A and B, a flow rate 1.5 μl/min, gradient of 5 to 95% solvent B over 30 min. The identification of the proteins from the MS/MS spectra was performed using protein identification software (Bioworks ver.3.1, Thermo Fisher Scientific) and UniProtKB/Swiss-Prot database (Release 48.8) which was preliminarily extracted by the species "human" (13361 entries). Peak list generation and database search were performed with the following parameters; mass tolerance for precursor ions, 2.5 amu; mass tolerance for fragment ions, 0.00 amu;, enzymatic cleavage position before lysine or arginine; number of missed cleavage sites permitted 2; fixed modification, carbamide-methylation (+57.02Da) for cysteine; variable modification, oxidation (+16Da) for methionine. The peptide sequences were filtered by peptide Cross Correlation (XC) score and delta Cn score. The threshold level of delta Cn score were >0.1 for peptide sequences from both measurement devices. The threshold level of XC score for each charge (+1/+2/+3) were >1.5/2.0/3.7 for LCQ and >1.9/2.2/3.7 for LTQ. Then correlation between observed in gel images and theoretical molecular weight and pI were

considered. When peptides matched multiple members of the protein family, the protein which had the most number of peptides matched to the amino acid sequence was selected. In case of equal numbers, both proteins were listed.

Western blotting and immunohistochemical analysis of macula

Five to fifteen micrograms of macula or peripheral retina homogenates were diluted in double volume of SDS buffer and separated by either 1D or 2D-PAGE followed by PVDF membrane transfer. Membranes were blocked with a Blocking Solution Concentrate (KPL, Gaithersburg, MD) and probed with one of the following primary antibodies (Abs): chicken Ab to human arrestin-C (GenWay Biotech, San Diego, CA), rabbit Ab to human Mn- superoxide dismutase (Mn-SOD) (upstate, Temecula, CA), rabbit Ab to human synuclein gamma (Novus Biologicals, Littleton, CO), rat Ab to human epidermal fatty acid binding protein (E-FABP) (R&D Systems, Inc. Minneapolis, MN), rabbit Ab to tropomyosin Br-1, Br-3 (CHEMICON International, Temecula, CA), mouse Ab to chicken tropomyosin TM311 (Abcam, Cambridge, UK), goat Ab to human heterogeneous nuclear ribonucleoproteins (hnRNPs) A2/B1 (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse Ab to human hnRNPs C1/C2 (abcam). The specific signals were detected with one of the following secondary antibodies: goat Ab to mouse IgG horseradish peroxidase

(HRP) conjugated (Jackson ImmunoResearch Laboratories, West Grove, PA), goat Ab to rabbit IgG HRP conjugated (Pierce, Rockford, IL), rabbit Ab to chicken/turkey IgG HRP conjugated (Zymed Laboratories, South San Francisco, CA), donkey Ab to goat IgG HRP conjugated (Jackson ImmunoResearch Laboratories), goat Ab to rat IgG HRP conjugated (Zymed Laboratories). The signals were made visible by chemiluminescence reactions and examined with a chemiluminescence imager (Lumi-Imager F1; Roche Diagnostics, Tokyo, Japan). The detection of Mn-SOD by 2D western blotting of macular or peripheral retina samples was made by 2D gel electrophoresis. Thirty micrograms of protein from each sample were separated using 7cm IPG strip at pH 3 to 10. The second dimensional separation was made on 12.5% polyacrylamide gel and then transferred to PVDF membranes. For quantitative comparisons of the band intensities of the western blots, the blots were scanned and measured by Lumi Analyst ver.3.1 (Roche Diagnostics).

Protein detection followed 1D western blotting

Enucleated eye from normal female cynomolgus monkey (age 13 yrs) were fixed in 10% neutralized and buffered formaldehyde solution at 4° C overnight and then dehydrated. The specimens were embedded in paraffin and serial sectioned at 4 μm thickness. The

specimens were treated for antigen retrieval by autoclaving in Target Retrieval Solution (Dako, Carpinteria, CA) for 20 min at 121° C. The sections were then blocked with Dako protein block (Dako) or skim milk or BSA in PBS. The primary antibodies were the same as used for the western blotting. For signal detection after rinsing off the primary antibodies, the sections were incubated with one of following secondary antibodies: Alexa 488 conjugated goat Ab mouse IgG (invitrogen, Tokyo, Japan), Alexa 568 conjugated goat anti-rabbit IgG (invitrogen), Alexa 488 conjugated donkey anti-goat IgG (invitrogen), Alexa 568 conjugated goat anti-chicken IgG (invitrogen), Alexa 488 conjugated goat anti-rat IgG (invitrogen). After rinsing off the antibodies, the sections were examined by confocal laser scanning microscope (Radiance 2100, Bio-Rad). The cell nuclei were stained with DAPI (4',6-diamino-2-phenylindole). To determine the location of the signals, one of sections was stained with hematoxylin and eosin.

Results

Identification of macula enriched proteins

Approximately 700 spots were detected in the macular and peripheral retinal tissues by 2D-gel (pH range 3-10; Fig. 1). Sixty percent of these spots were found in both

samples. The gel spots indicating high expression of proteins are listed in Table 1. Forty eight known proteins from earlier proteomics studies of the retina were identified (12-14, 29, 30). Fifteen spots were specific to the macula. The neurofilament triplet L protein identified in this study has not been previously reported by proteomic approach but by previous SAGE analysis (6). To obtain a further separation, the pH range of the 2D-gel electrophoresis was narrowed to three ranges; 4-7, 5-8, and 7-10 (Fig. 2-4). Under these conditions, 40 spots specific to macula were further detected and 26 proteins were identified (Table 2). To obtain the relative expression levels of the identified proteins, western blotting analysis was performed.

Quantification of macula enriched proteins

Western blotting was performed on the following seven proteins identified by mass spectrometry; arrestin-C, Mn-SOD, γ-synuclein, E-FABP, tropomyosin1a chain, nhnRNPs A2/B1, and hnRNPs C1/C. Arrestin-C showed a 1.5-fold higher expression in the macula than the peripheral retina (Fig. 5-1). Arrestin-C has been previously identified as a cone photoreceptor specific protein (31), and thus served as a positive control for this study. Mn-SOD, an anti oxidative enzyme located in mitochondria (32), showed equal expression in the macular and peripheral retina (Fig. 5-2). For Mn-SOD, 2D-gel western

blotting was performed to observe the distribution of spots. Figure 6 showed an additional spot in the acidic position (arrow) on the macula membrane compared with peripheral retina. γ-synuclein, a protein known to be up-regulated in cancer cells (33), showed 2.9-fold higher expression in the macula. E-FABP, a reactive lipid scavenger (34), showed 1.9-fold higher expression in the macula. Tropomyosin1a chain Br-1 and Br-3 showed 1.8-fold higher expression in the macula than in the peripheral retina, while tropomyosin1a chain TM311 showed 8-fold higher expression in the macular (Fig. 5-5 & 5-6). Tropomyosin1a chain has been reported in many isoforms by alternative splicing (35). MS/MS data from spot M4 (Fig.1) identified peptide sequence CAELEEELK (Table 2), which corresponded to isoform 1 (skeletal muscle type) or isoform 5 (brain type, TMBr-3) of tropomyosin1a chain in the UniProtKB/Swiss-Prot database.

Based on these data, western blotting was performed using two antibodies for tropomyosin1a chain isoform 1 and 5. The anti-tropomyosin antibody TM311 detects 19 amino acids in exon 1a of tropomyosin gene family in mammalian tissues, viz., alpha-, beta-, gamma-, delta- tropomyosin, including the skeletal muscle type but it does not detect brain type (TMBr-3). The other antibodies used were specific to the brain isoforms TMBr-1 and TMBr-3. hnRNPs A2/B1 had 1.5-fold higher expression in the macula, while hnRNPs C1/C2, had 2.6-fold higher expression in the macula than peripheral retina (Fig.

5-7 & 5-8). hnRNPs A2/B1 is known to be up-regulated in carcinoma cells (36) and hnRNPs C1/C2 for amino acid modification by oxidative stress (37). hnRNPs A2/B1 and hnRNPs C1/C2 products are known as alternative splicing variants. A unique amino acid sequence of hnRNP B1 isoform was identified from spot M37 and M38 but other hnRNPs A2/B1 and all hnRNPs C1/C2 spots contained only common amino acid sequences of each variant.

Tissue localization of macula enriched proteins

To determine the location of the 7 proteins in the macula, immunohistochemistry was performed using antibody against each protein (Fig. 7). Arrestin-C was detected in photoreceptor outer segments and outer plexiform layer (Fig. 7B) as reported (31). γ-synuclein was located in the retinal ganglion cells and the nerve fiber layer. E-FABP was detected throughout the sensory retina except the photoreceptors and RPE cells (Fig. 7E). Tropomyosin Br-1 and Br-3 were located in the photoreceptor inner segments and outer plexiform layer (Fig. 7F), while TM311 was detected in the choroidal layer (Fig. 7G). hnRNPs A2/B1 and hnRNPs C1/C2 were located in the nucleus of a number of retinal cells including the cells in the retinal ganglion cell layer, the inner nuclear layer, the outer nuclear layer, and the RPE layer. However, a difference in the signal intensity was