(rs11200638), and other behavioral, nutritional, and environmental factors. Further replication and detailed experiments are needed.

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Suppression of Drusen Formation by Compstatin, a Peptide Inhibitor of Complement C3 activation, on Cynomolgus Monkey with Early-Onset Macular Degeneration

Zai-Long Chi[1], Tsunehiko Yoshida[2], John D. Lambris[3], and Takeshi Iwata[4]

[1]Division of Molecular & Cellular Biology, National Institute of Sensory Organs, National Hospital Organization Tokyo Medical Center, Tokyo, Japan, chizai-long@kankakuki.go.jp

[2]Division of Molecular & Cellular Biology, National Institute of Sensory Organs, National Hospital Organization Tokyo Medical Center, Tokyo, Japan, yoshidatsunehiko@kankakuki.go.jp

[3]Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA, USA, lambris@mail.med.upenn.edu

[4]Division of Molecular & Cellular Biology, National Institute of Sensory Organs, National Hospital Organization Tokyo Medical Center, Tokyo, Japan, iwatatakeshi@kankakuki.go.jp

Abstract. For the past ten years, number of evidence has shown that activation of complement cascade has been associated with age-related macular degeneration (AMD). The genome wide association study in American population with dominantly dry-type AMD has revealed strong association with single nucleotide polymorphism (SNP) of complement genes. Protein composition of drusen, a deposit observed in sub-retinal space between Bruch's membrane and retinal pigment epithelial (RPE), contains active complement molecules in human and monkey. These evidences have leaded us to consider the possibility of suppressing complement cascade in the retina to delay or reverse the onset of AMD. To test is hypothesis we used the C3 inhibitor Compstatin on primate model with early-onset

macular degeneration which develop drusen in less than 2 years after birth. Our preliminary result showed drusen disappearance after 6 month of intravitreal injection.

1 AMD and association of complement related genes

The most prevalent eye disease for elderly Europeans and Americans is AMD. AMD is a blinding disorder characterized by a marked decrease in central vision associated with retinal pigment epithelial (RPE) atrophy with or without choroidal neovascularization (CNV). The non-neovascular type is called the dry-type AMD and includes more than 80% of the cases, and the neovascular type is called the wet-type AMD which is progressive with a higher probability of blindness. In some cases of CNV, the new vessels penetrate Bruch's membrane and pass into the subretinal space. The progressive impairment of the RPE and damage to Bruch's membrane and choriocapillaris results in retinal atrophy and photoreceptor dysfunction.

Genetic, behavioral, and environmental factors are believed to be involved for the onset of this disease. The prevalence of AMD differs considerably among the different ethnic groups, but the incidence increases with age in all groups. Epidemiological studies have shown that genetic factor play critical role for AMD. However, only a small proportion of the families with AMD show Mendelian inheritance, and the majority of the individuals inherit AMD in a complex multi-gene pattern. With the help of the haplotype marker project (HapMap Project), genome wide scanning has identified at least 13 loci linked to AMD on different chromosomes (Iyengar et al. 2004; Schick et al. 2003; Majewski et al. 2003). Other risk factors such as cigarette smoking, obesity, hypertension, and atherosclerosis are also associated with the disease.

Recently, a polymorphism of complement factor H (CFH) gene (Y402H) was shown to be associated with an increased risk for AMD (Klein

et al. 2005; Edwards et al. 2005; Haines et al. 2005; Hageman et al. 2005). These results were confirmed in many of the countries with large Caucasian populations but not in Japan (Okamoto et al. 2006; Gotoh et al. 2009). This gene is located on chromosome 1q25-31 where one of the candidate loci was identified by whole genome association studies by linkage markers. Another recent study reported that a haplotype association of tandemly located complement 2 and factor B (Gold et al. 2006) was protective and C3 (Yates et al. 2007) as risk for AMD. HTRA1, a serine protease 11 was recently discovered to be strongly associated with AMD (Yang et al. 2006; Dewan et al. 2006). Unlike the CFH, our study shows strongly association with this gene for Japanese AMD patients (Yoshida et al. 2007). This difference of gene association is probably related to the difference of AMD type dominant in each country. Our genome wide association study on Japanese population with typical wet-type AMD and polypoidal choroidal vasculopathy (PCV) shows significant association at p-value of 10^{-14} and 10^{-7} respectively for ARMS2/Htra1 locus. However when much lower associated SNPs of CFH or C3 or combined the odds ratio significantly increased (Goto, Akahori, et al. 2009)

2 Activated complement component in drusen

The early stage of the dry-type AMD is characterized by thickening of Bruch's membrane, aggregation of pigment granules, and increasing numbers of drusen. Drusen are small yellowish-white deposits that are composed of lipids, proteins, glycoproteins, and glycosaminoglycans. They accumulate in the extracellular space and the inner aspects of Bruch's membrane. Drusen are not directly associated with visual loss but represent a risk factor for dry-type AMD. The classification of hard and soft drusen is based on their size, shape, and color; hard drusen are yellowish with diameters <50 μm and are found in eyes that are less likely to progress to advanced stages of the disease,

while soft drusen are darker yellow and larger in size, and are found in eyes more likely to progress to more advanced stages of AMD.

Both immunohistochemistry and proteomic techniques have shown that drusen are composed of molecules that mediate inflammatory and immune processes (Russell et al. 2000; Mullins et al. 2000). These molecules include components of the complement pathway and modulators of complement activation, viz., vitronectin, clusterin, membrane cofactor protein, and complement receptor-1. In addition, molecules triggering inflammation, amyloid P component, α1-antitrypsin, and apolipoprotein E, were identified in drusen. Cellular debris from macrophages, RPE cells, and choroidal dendritic cells has been identified in drusen. Additional proteins such as crystallins, EEFMP1, and amyloid-beta have been also found in drusen. The presence of immunoreactive proteins and the oxidative modifications of many proteins in drusen imply that both oxidation and immune functions are involved in the pathogenesis of AMD. Finding of these molecules suggest that complement activation triggers innate immune responses in the subretinal space.

3 Cynomolgus monkey with early-onset macular degeneration

Over the past years non-human primates with well-defined fovea has been the target for AMD research. A monkey with macular degeneration was first described by Stafford et al in 1974. They reported that 6.6 % of the elderly monkeys they examined showed pigmentary disorders and drusen-like spots (Stafford et al. 1984). We also observed at approximately the same rate of disorder in elderly cynomolgus monkeys in the Philippines primate facility (SICONBREC) (Umeda et al. 2005). El-Mofty et al reported that the incidence of maculopathy was 50% in a colony of rhesus monkeys at the Caribbean Primate Research Center of the University of Puerto Rico (El-Mofty et al. 1978). In 1986, a single cynomolgus monkey (*Macaca*

fascicularis) with large number of small drusen in the macula was found in Tsukuba Primate Research Center at Tsukuba City, Japan (Nicolas et al. 1996; Nicolas et al. 1996; Suzuki et al. 2003). This single affected monkey has been bred to a large pedigree of more than 300 monkeys. Drusen are observed in the macula as early as two year after birth, and the number increase and spread toward the peripheral retina throughout life. Histological abnormalities of the retina and abnormal electroretinogram (ERG) were observed in sever case showing physiological dysfunction of the macula.

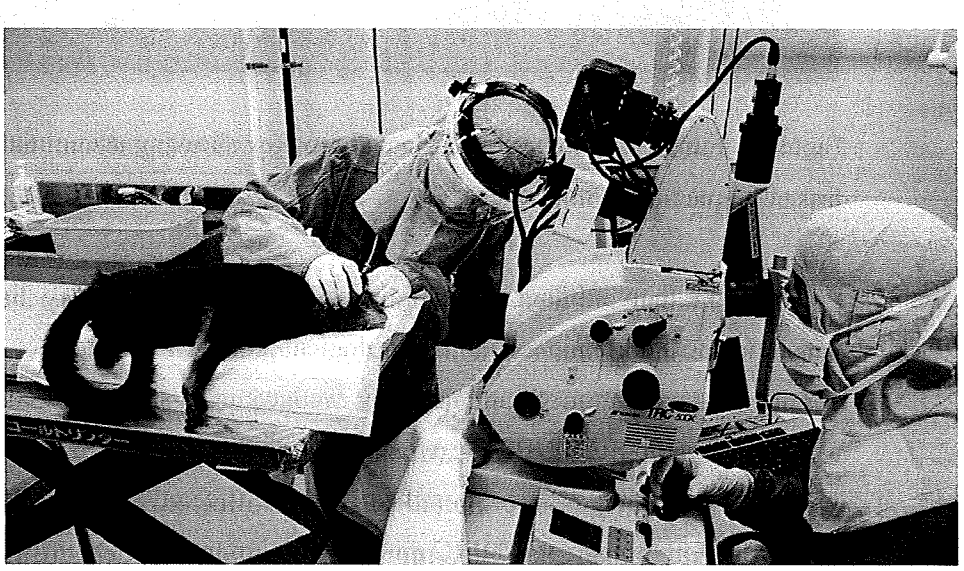

Figure 1. Fundus photography of affected monkey at TPRC.

Immunohistochemical and proteomic analyses of the drusen from these monkeys showed that the drusen were very similar to those in other monkeys with aged macular degeneration sporadically found in older monkeys and also with human drusen (Umeda et al. 2005; Umeda et al. 2005; Ambati et al. 2003). These observations have shown that TPRC monkeys produce drusen that are biochemically similar to those in human AMD patients, but the development of the drusen occurs at an accelerated rate.

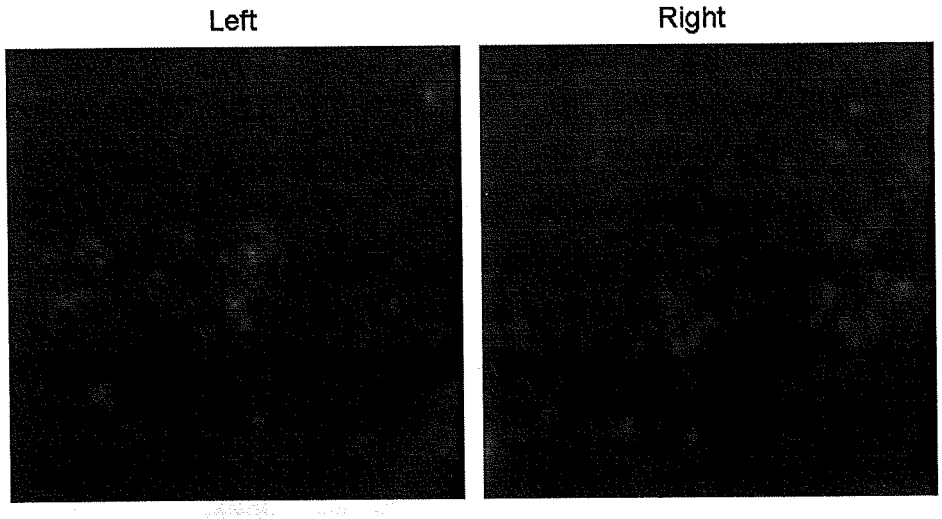

Figure 2. Fundus photograph of affected monkey showing accumulation of drusen in macula of both eyes.

More than 240 loci are being investigated to try to identify the disease causing gene and to understand the biological pathways leading to complement activation. Simultaneously, we have been studying a colony of aged monkeys in SICONBREC, which develop drusen after 15 years of birth. Drusen components of these sporadically found affected monkeys were compared with human and TPRC monkeys by immunohistochemistry and proteomic analysis using ion spray mass spectrometer. Significant finding was that drusen contained protein molecules that mediate inflammatory and immune processes. These include immunoglobulins, components of complement pathway, and modulators for complement activation (e.g., vitronectin, clusterin, membrane cofactor protein, and complement receptor-1), molecules involved in the acute-phase response to inflammation (e.g., amyloid P component, α1-antitrypsin, and apolipoprotein E), major histocompatibility complex class II antigens, and HLA-DR antigens (Umeda et al. 2005). Cellular components have also been identified in drusen,

including RPE debris, lipofuscin, and melanin, as well as processes of choroidal dendritic cells, which contribute to the inflammatory response. The presence of immunoreactive proteins and oxidative modified proteins implicate both oxidation and immune functions in the pathogenesis of affected monkeys.

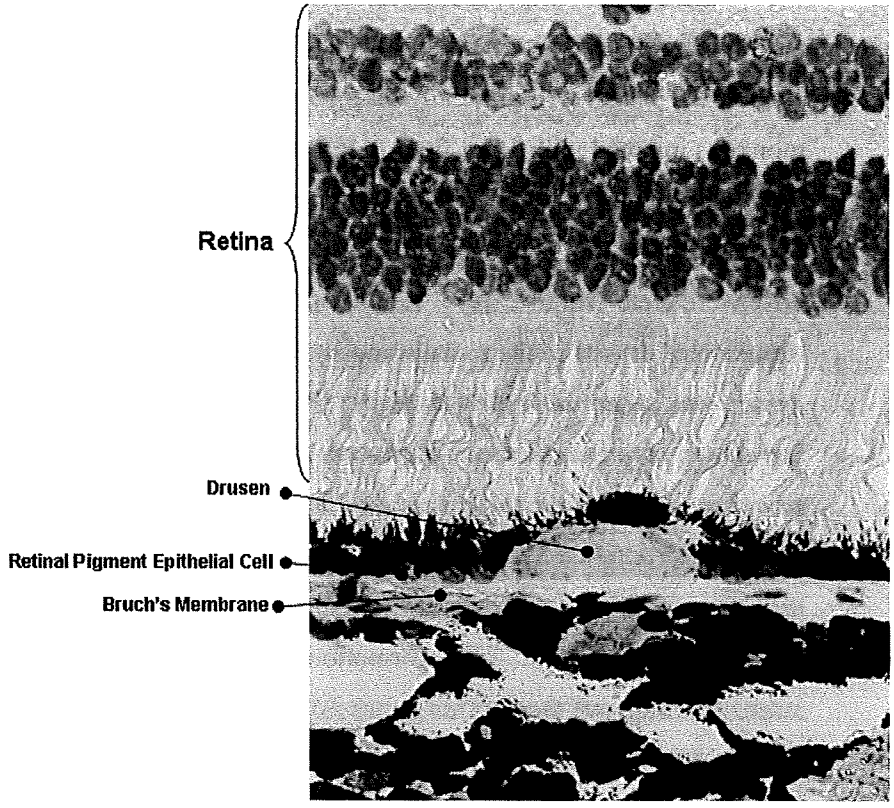

Figure 3. Retinal histological section of affected monkey showing the accumulation of drusen.

4 Suppression and reversal of drusen formation by compstatin

To test the effect of long term suppression of complement activation in the retina, an cyclic analogue (Ac-I[CV(1MeW)QDWGAHRC]T-NH2) of the small cyclic synthetic peptide compstatin (Katragadda,M. et al. 2006) was

intravitreally injected into 8 affected monkeys at different dose and intervals. Four affected monkeys were injected at 1 mg dose at one month interval while other four affected monkeys at 50 μg dose at one week interval. Both 1 mg or 50 μg dose were dissolved in 100 μl of saline solution, filtrated and intravitreally injected using 30G needle.

Due to the unique molecular characteristic of compstatin, immediately after injection, compstatin precipitate and form gel-like structure in the vitreous. This gel will gradually dissolve and disappear after 6 month. Four monkeys injected with 1 mg for 3 month developed significant opacity to the point where fundus observation was impossible. These monkeys were halted for further injection. On the other hand, vitreous of 4 monkeys with 50 μg dose were clear within 2 days. After 6 month of injection we noticed diffusion of drusen in the macula and by 9 month partial disappearance of drusen was observed in all 4 monkeys (Fig. 4). This preliminary experiment has shown reversal of drusen formation by suppression of complement activation. To explain this reversal phenomenon, which has not been observed in untreated affected monkeys, will require further experiments including identification of disease causing gene and pathway leading to complement activation. The information should benefit for development of improved drug and therapy for future AMD prevention.

All experimental procedures for this primate study were approved by the Animal Welfare and Animal Care Committee of the TRPC and the Experimental Animal Committee of the National Tokyo Medical Center. The facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). Monkeys were routinely examined for physical and ophthalmic conditions by veterinarians and by ophthalmologists, respectively.

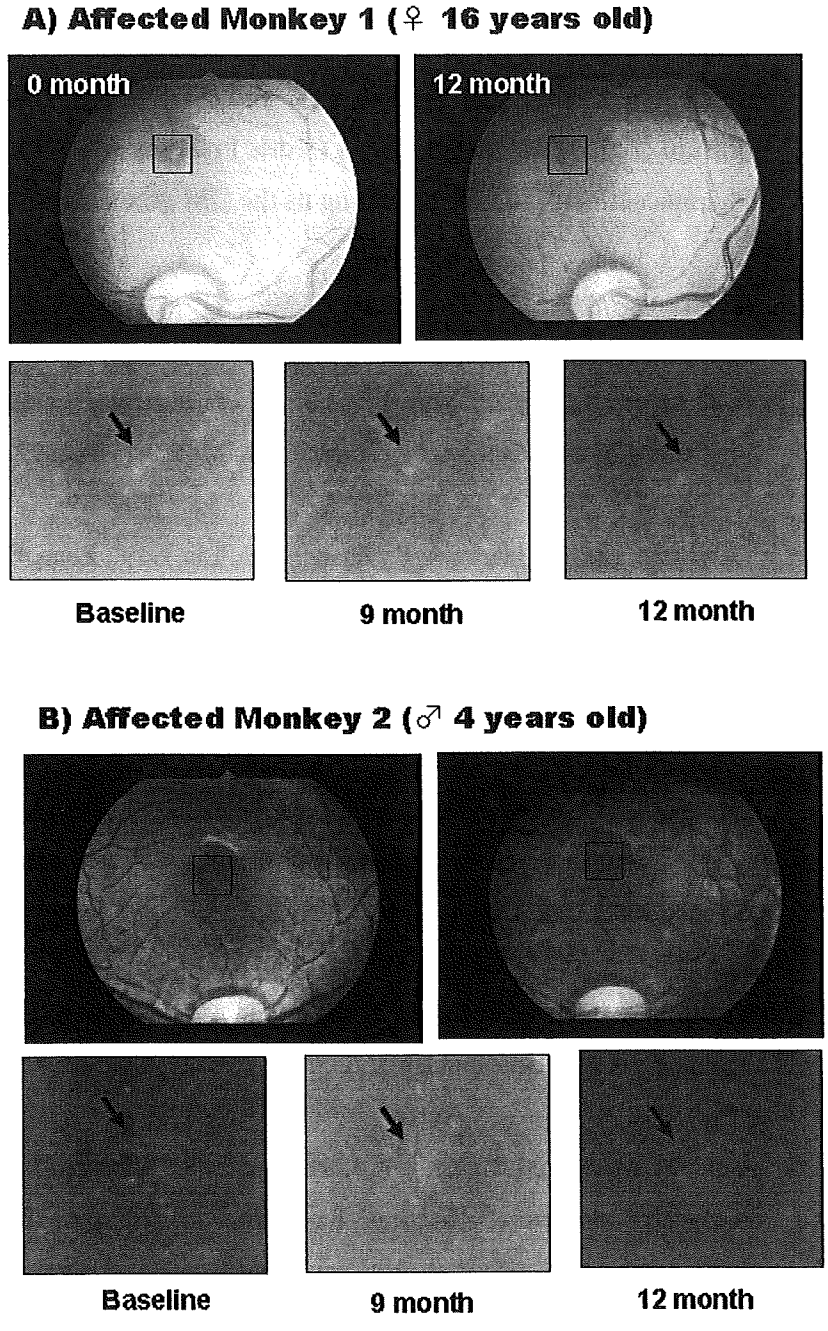

Figure 4. Suppression and reversal of drusen formation after 9 month of intravitreal injection of 50 µl compstatin at one week interval.

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—Original—

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

Haru OKAMOTO[1,2], Shinsuke UMEDA[1], Takehiro NOZAWA[3], Michihiro T. SUZUKI[4], Yasuhiro YOSHIKAWA[5], Etsuko T. MATSUURA[6], and Takeshi IWATA[1]

[1]Division of Molecular & Cellular Biology, National Institute of Sensory Organs, National Hospital Organization Tokyo Medical Center, 2–5–1 Higashigaoka, Meguro-ku, Tokyo 152-8902, Japan, [2]Department of Advanced Biosciences, Ochanomizu University, 2–1–1 Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan, [3]Analytical Instrument Division, AMR Inc., 2–13–18 Nakane, Meguro-ku, Tokyo 152-0031, Japan, [4]The Corporation for Production and Research of Laboratory Primates, 1–1 Hachimandai, Tsukuba-shi, Ibaraki 305-0843, Japan, [5]Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan, and [6]The Natural/Applied Sciences Division, Ochanomizu University, 2–1–1 Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan

Abstract: The central region of the primate retina is called the macula. The fovea is located at the center of the macula, where the photoreceptors are concentrated to create a neural network adapted for high visual acuity. Damage to the fovea, e.g., by macular dystrophies and age-related macular degeneration, can reduce central visual acuity. The molecular mechanisms leading to these diseases are most likely dependent on the proteins in the macula which differ from those in the peripheral retina in expression level. To investigate whether the distribution of proteins in the macula is different from the peripheral retina, proteomic analyses of tissues from these two regions of cynomolgus monkeys were compared. Two-dimensional gel electrophoresis and mass spectrometry identified 26 proteins that were present only in the macular gel spots. The expression levels of five proteins, cone photoreceptor specific arrestin-C, γ-synuclein, epidermal fatty acid binding protein, tropomyosin 1α chain, and heterogeneous nuclear ribonucleoproteins A2/B1, were significantly higher in the macula than in the peripheral retina. Immunostaining of macula sections by antibodies to each identified protein revealed unique localization in the retina, retinal pigment epithelial cells and the choroidal layer. Some of these proteins were located in cells with higher densities in the macula. We suggest that it will be important to study these proteins to determine their contribution to the pathogenesis and progression of macula diseases.

Key words: 2D-gel electrophoresis, macula, mass spectrometry, retina

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Address corresponding: T. Iwata, Division of Molecular & Cellular Biology, National Institute of Sensory Organs, National Hospital Organization Tokyo Medical Center, 2–5–1 Higashigaoka, Meguro-ku, Tokyo 152-8902, Japan

Introduction

The macula is an oval-shaped, highly pigmented spot near the central region of the primate retina. It is approximately 2.0 mm in diameter in humans [31] and 1.0 mm in macaque monkeys [36–38]. The fovea is located at the center of macula where the retinal thickness is reduced to approximately 0.1 mm and consists of only the retinal pigment epithelium (RPE), photoreceptor layer, external limiting membrane, outer nuclear layer, outer plexiform layer, and inner limiting membrane [30]. The cone density in the foveal pit is the highest in the retina, and rods, retinal ganglion cells (RGCs), and blood vessels are not present. These cones are connected to large numbers of RGCs, which are highly dense at the parafovea [31]. The cone-dense fovea mediates high-acuity central vision, and any damage to the macula can lead to severely depressed central visual acuity as observed in patients with macular dystrophies and age-related macular degeneration (AMD).

Because of the unique cellular organization of the macula, investigators have performed comprehensive gene expression studies of the macula and peripheral retina using DNA microarray analysis or Serial Analysis of Gene Expression (SAGE). Sharon *et al.* used SAGE to show that several genes are preferentially expressed in the human macula and RPE. Most of these genes are associated with the function of the RGCs, and were presumably detected because of the high density of RGCs in the macula [35]. Bowes Rickman *et al.* also performed SAGE on human retinas and isolated RPE cells and identified genes that are abundantly expressed in cones, RGCs, and RPE cells [3]. Ishibashi *et al.* performed 4 K DNA microarray analysis on RPE cells from the macula and reported five differentially expressed genes which were confirmed by real-time PCR [18]. Recently, Radeke *et al.* [32] and van Soest *et al.* [48] used 22 K DNA microarray analyses and identified a number of genes that were differentially expressed in the macula and peripheral retina. In each study, five of these genes were found to be highly expressed in RPE cells in the macula. van Soest *et al.* showed by immunohistochemistry that the WAP four-disulfide core domain 1, one of the highly expressed proteins, is present in the RPE cells in the macula. However, the expression level of the mRNAs does not always correlate with the expression levels of the proteins.

Recent technical advances in proteomics allow the direct determination of the protein profile of body fluids and tissue homogenates. Proteomic analyses of the retina were first performed by Nishizawa *et al.* [28], and several groups have catalogued the retinal proteins using single or two-dimensional (2D) gel electrophoresis followed by mass spectrometry (MS) analysis [1, 5, 50]. Ethen *et al.* examined cadaver eyes with AMD by proteomic analyses and reported that the expression of proteins changed with the progression of AMD, and the changes in the macula were different from those in the peripheral retina [10]. These findings indicate that the macular region of the retina is different from the peripheral retina not only in its morphology but also in its protein content.

Proteomic studies of the macula are difficult to perform because of the lack of fresh human eyes, and small sample size of the macula. To overcome these problems, we selected non-human primate eyes of the cynomolgus monkey (*Macaca fascicularis*). The retina and visual system of macaque monkeys are quite similar to those of humans [14, 29], and monkeys with characteristics of macular diseases have been reported by many investigators [9, 16, 19, 25, 39, 40] as well as our previous studies [26, 27, 44–47]. Thus, the purpose of this study was to identify proteins present at high levels in the macula to better understand the biology of this unique tissue. To accomplish this task, we performed proteomic analyses on retinal tissues obtained from the macular region and the periphery for comparison.

Materials and Methods

Preparation of cynomolgus monkey eyes

All experiments on monkeys were approved by the Animal Ethics Committee of the Tsukuba Primate Research Center (TPRC) and were conducted in accordance with 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). Other tissues from these animals were used by other research groups at the TPRC. Three-millimeter-diameter pieces of macular and peripheral retina containing neural retina, RPE and choroidal layer were punched out and frozen until use. The proteins extracted from the tissues of eight eyes were pooled for the 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, USA)]. After centrifugation for 15 min × 3 at 14,000 rpm (20,800 × *g*), the supernatant was collected. The lysate was precipitated using Ready Prep 2D cleanup kit (Bio-Rad) and redissolved in sample buffer. The protein concentration was determined with the RC-DC protein assay kit (Bio-Rad) according to the manufacturer's instruction. Protein samples (300 μg) were separated by isoelectric focusing (IEF) using 17-cm immobilized pH gradient (IPG) strips. After 12 to 16 h of rehydration at 20°C, the IEF sample 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 h and held until 60,000 V-h was reached. Immediately after the IEF, the IPG strips were stored at –20°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. The equilibrated IPG strips were electrophoresed for the second dimension using 12% acrylamide gels. Two dimensional gel electrophoresis was performed at four different pH ranges, viz., pH 3–10, 4–7, 5–8, and 7–10. After the 2D gel electrophoresis, the proteins were stained with SYPRO Ruby (Bio-Rad). The images for the macula and peripheral retina were compared with ImageMaster 2D Platinum ver.5.0 (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) followed by visual inspection. The gel spots numbered in Fig. 1 were excised. Two dimensional gels of peripheral retina were stained with Bio-safe Coomassie (Bio-Rad). Then, 46 spots from the Coomassie-stained gel were excised (Fig. 1, Peripheral Coomassie).

In gel digestion and LC-MS/MS analyses

Each gel piece was cut into 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 sequencing grade trypsin solution (12.5 ng/μl; Promega, Madison, WI, USA) 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 (liquid chromatography)-MS/MS. LC-MS/MS was performed with a combined Paradigm MS4 (Michrom BioResources, Auburn, CA, USA) 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 in nano column (AMR Inc.) with solvent A (2% acetonitrile/0.1% formic acid) and B (90% acetonitrile/0.1% formic acid) at 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" (13,361 entries). Peak list generation and database searches 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, after lysine or arginine; number of missed cleavage sites permitted, 2; fixed modification, carbamide-methylation (+57.02 Da) for cysteine; variable modification, oxidation (+16 Da) for methionine. The peptide sequences were filtered by delta Cn score and peptide Cross Correlation (XC) score. The threshold level of delta Cn scores was >0.1 for peptide sequences from both measurement devices. The threshold levels of XC scores 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, the correlations between the observed in gel images and the 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 that matched 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 retinal homogenates were diluted in a double volume of SDS buffer and separated by 1D-PAGE followed by transfer to PVDF (polyvinylidene difluoride) membrane. Membranes were blocked with blocking solution (Blocking Solution Concentrate, KPL, Gaithersburg, MD, USA), skim milk, or BSA dissolved in PBS and probed with one of the following primary antibodies (Abs): chicken Ab to human arrestin-C (GenWay Biotech, San Diego, CA, USA), rabbit Ab to human synuclein gamma (Novus Biologicals, Littleton, CO, USA), rat Ab to human epidermal fatty acid binding protein (E-FABP) (R&D Systems, Minneapolis, MN, USA), rabbit Ab to tropomyosin Br-1, Br-3 (CHEMICON International, Temecula, CA, USA), mouse Ab to chicken tropomyosin TM311 (Abcam, Cambridge, UK), and goat Ab to human heterogeneous nuclear ribonucleoproteins (hnRNPs) A2/B1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The specific signals were detected with one of the following secondary antibodies: horseradish peroxidase (HRP)-conjugated goat Ab to mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), HRP-conjugated goat Ab to rabbit IgG (Pierce, Rockford, IL, USA), HRP-conjugated rabbit Ab to chicken/turkey IgG (Zymed Laboratories, South San Francisco, CA, USA), HRP-conjugated donkey Ab to goat IgG (Jackson ImmunoResearch Laboratories), HRP-conjugated goat Ab to rat IgG (Zymed Laboratories). The signals were made visible by chemiluminescence reactions and examined with a chemiluminescence imager (Lumi-Imager F1; Roche Diagnostics, Tokyo, Japan). An enucleated eye from a normal female cynomolgus monkey (age 13 years) was fixed in 10% neutralized and buffered formaldehyde solution at 4°C overnight and then dehydrated. The specimens were embedded in paraffin and serially sectioned at 4 μm thickness. The specimens were treated for antigen retrieval by autoclaving in Target Retrieval Solution (Dako, Carpinteria, CA, USA) 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, and rabbit Ab to human platelet/endothelial cell adhesion molecule (PECAM1) (Proteintech Grroup, Chicago, IL, USA). For signal detection after rinsing off the primary antibodies, the sections were incubated with one of following secondary antibodies: Alexa 488-conjugated goat Ab to anti-mouse IgG, Alexa 568-conjugated goat Ab to anti-mouse IgG, Alexa 488-conjugated goat Ab to anti-rabbit IgG, Alexa 568-conjugated goat Ab to anti-rabbit IgG, Alexa 488-conjugated donkey Ab to anti-goat IgG, Alexa 568-conjugated goat Ab to anti-chicken IgG, and Alexa 488-conjugated goat Ab to anti-rat IgG (all secondary antibodies from Invitrogen, Tokyo, Japan). 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 the 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 in the 2D gel stained with SYPRO Ruby (pH range 3–10; Fig. 1A). Sixty percent of these spots were found in both samples. Butt *et al.* have described the difficulties of IEF separation by RNAlater contaminations [4]. Our samples were de-