Table 1. Proteins identified in 46 spots of Coomassie-stained gel

Spot No.[a)]	Protein name	Database accession No.[b)]	MW (kDa)[c)]	pI[c)]	Sequence coverage (%)	No. of peptide
1	Heat shock protein HSP 90-alpha	P07900	84.5	4.94	10.53	7
1	Heat shock protein HSP 90-beta	P08238	83.1	4.97	6.09	4
2	Heat shock cognate 71 kDa protein	P11142	70.9	5.37	29.41	17
2	Vacuolar ATP synthase catalytic subunit A, ubiquitous isoform	P38606	68.3	5.35	20.75	10
2	Lamin B2	Q03252	67.7	5.29	17.67	9
2	Heat shock 70 kDa protein 1	P08107	70 1	5.48	16.54	9
2	Stress-70 protein, mitochondrial	P38646	73.7	5.87	9.57	5
3	Stress-70 protein, mitochondrial	P38646	73 7	5.87	8.25	4
3	Serum albumin	P02768	69.4	5.92	6.9	4
4	Serum albumin	P02768	69.4	5.92	6.9	3
5	Serum albumin	P02768	69.4	5.92	7.39	4
6	Serum albumin	P02768	69.4	5.92	3.94	2
7	Serotransferrin	P02787	77.1	6.81	3.72	2
8	Neurofilament triplet L protein	P07196	61.4	4.64	23.99	13
9	Calreticulin	P27797	48.1	4.29	15.59	6
10	Protein disulfide-isomerase	P07237	57.1	4.76	5.12	2
11	60 kDa heat shock protein, mitochondrial	P10809	61 1	5 7	15.36	8
11	Pyruvate kinase, isozymes M1/M2	P14618	57.8	7.95	3.58	2
12	Vimentin	P08670	53.5	5.06	39.57	15
13	Vacuolar ATP synthase subunit B, brain isoform	P21281	56.5	5.57	5.09	2
14	Vacuolar ATP synthase subunit B, brain isoform	P21281	56.5	5.57	7.24	3
15	Tubulin alpha-3 chain	Q71U36	50 1	4.94	17.29	7
15	Tubulin alpha-1 chain	P68366	49.9	4.95	14.96	6
16	S-arrestin	P10523	45.1	6.14	3.7	1
17	S-arrestin	P10523	45 1	6 14	19.01	7
18	S-arrestin	P10523	45.1	6 14	22.96	8
19	Tubulin beta-2C chain	P68371	49.8	4.79	19.55	8
19	Tubulin beta-2 chain	P07437	49.7	4.78	18.92	8
19	Tubulin beta-3 chain	Q13509	50.4	4.83	16.89	7
19	Tubulin beta-6 chain	Q9BUF5	49.9	4.77	11.88	5
20	ATP synthase beta chain, mitochondrial	P06576	56.6	5.26	25.9	8
21	Gamma-enolase	P09104	47.1	4.91	12.47	5
22	Eukaryotic initiation factor 4A-II	Q14240	46.4	5.33	16.22	5
22	Eukaryotic initiation factor 4A-I	P60842	46.2	5.32	12.56	4
23	Alpha-enolase	P06733	47	6.99	31.41	9
24	Alpha-enolase	P06733	47	6.99	24.71	9
25	Alpha-enolase	P06733	47	6.99	14.32	6
26	Alpha-enolase	P06733	47	6.99	22.86	8
27	Actin, cytoplasmic 1	P60709	41.7	5.29	16.53	5
27	Actin, cytoplasmic 2	P63261	41.8	5.31	16.53	5
27	Actin, gamma-enteric smooth muscle	P63267	41.9	5.31	11.7	4
27	Actin, aortic smooth muscle	P62736	42	5.24	11.67	4
28	Creatine kinase B-type	P12277	42.6	5.34	14 17	4
29	Glutamine synthetase	P15104	41.9	6.42	6.45	3
30	Glutamine synthetase	P15104	41.9	6.42	24.73	8
31	Glutamine synthetase	P15104	41.9	6.42	6.45	2
32	L-lactate dehydrogenase B chain	P07195	36.5	5.72	7.51	2
33	L-lactate dehydrogenase B chain	P07195	36.5	5.72	27.03	7
34	L-lactate dehydrogenase B chain	P07195	36.5	5.72	39.04	11
35	Cellular retinaldehyde-binding protein	P12271	36.3	4.98	22.15	6
36	Inorganic pyrophosphatase	Q15181	32.7	5.54	11 76	3
36	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1	P62873	37.2	5.6	6.49	2
37	Inorganic pyrophosphatase	Q15181	32.7	5.54	14.88	4
37	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1	P62873	37.2	5.6	11.5	4
38	Malate dehydrogenase, mitochondrial	P40926	35.5	8.92	19.82	6
38	Glyceraldehyde-3-phosphate dehydrogenase	P04406	35.9	8.58	10 78	3
39	14-3-3 protein epsilon	P62258	29.2	4.63	14.51	3
40	14-3-3 protein zeta/delta	P63104	27.7	4 73	14.69	3
40	14-3-3 protein theta	P27348	27.8	4.68	14.69	3
40	14-3-3 protein gamma	P61981	28.2	4.8	13.82	3
41	Recoverin	P35243	23	5.06	10.05	2
42	ATP synthase delta chain, mitochondrial	P30049	17.5	5.38	5.36	1
43	Alpha crystallin A chain	P02489	19.9	5.77	16 76	3
44	Hemoglobin beta subunit	P68871	15.9	6.81	22.6	3
45	Hemoglobin beta subunit	P68871	15.9	6.81	22.6	3
46	Hemoglobin alpha subunit	P69905	15.1	8 73	23.4	3

[a)]Spot numbers correspond to the numbers on gel images in Fig. 1 (Peripheral-Coomassie). [b)]Accession No. corresponds to UniProtKB/Swiss-prot database. [c)]MW and pI are theoretical scores.

Table 2. Proteins identified in 40 spots detected only in macular gels

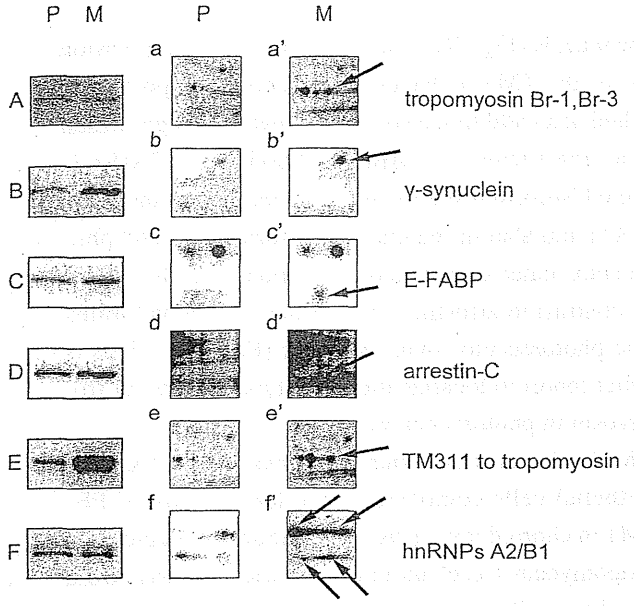
Spot No [a]	Protein name	Database accession No.[b]	MW (kDa)[c]	pI[c]	Sequence coverage (%)	No. of peptide
M1	Pyruvate kinase, isozymes M1/M2	P14618	32.7	4.69	18.11	8
M2	Tropomyosin 1 alpha chain	P09493	32 7	4.69	13.73	5
M2	Heterogeneous nuclear ribonucleoproteins C1/C2	P07910	33.7	4.95	16.67	4
M3	Transaldolase	P37837	37.5	6.36	16.62	5
M3	3'(2'),5'-bisphosphate nucleotidase 1	O95861	33.4	5.46	10.06	3
M4	Poly(rC)-binding protein 1	Q15365	37.5	6.66	16.85	5
M5	Crk-like protein	P46109	33.8	6.26	7.59	2
M6	Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	37.4	8.97	4.53	1
M7	Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	37.4	8.97	11.9	3
M8	Voltage-dependent anion-selective channel protein 2	P45880	38 1	6.32	9.8	3
M9	Voltage-dependent anion-selective channel protein 1	P21796	30.6	8.63	7.8	2
M10	Voltage-dependent anion-selective channel protein 1	P21796	30.6	8.63	15.6	3
M11	Endoplasmic reticulum protein ERp29	P30040	29	6.77	27.59	6
M12	Guanylate kinase	Q16774	21.6	6.11	13.27	2
M13	Guanylate kinase	Q16774	21.6	6.11	19.39	3
M14	Gamma-synuclein	O76070	13.3	4.97	12.6	1
M15	Fatty acid-binding protein, epidermal	Q01469	15	6.8	21.64	3
M16	Arrestin-C	P36575	42.8	5.53	14.95	4
M17	Arrestin-C	P36575	42.8	5.53	4.64	1
M18	Isocitrate dehydrogenase [NAD] subunit alpha	P50213	39.6	6.46	22.4	7
M18	Transaldolase	P37837	37.5	6.36	15.73	5
M19	Tropomyosin 1 alpha chain	P09493	32.7	4.69	14.79	3
M19	Heterogeneous nuclear ribonucleoproteins C1/C2	P07910	33 7	4.95	12.75	3
M20	Pyruvate dehydrogenase E1 component beta subunit	P11177	39.2	6.2	31.75	8
M21	Glucose-6-phosphate 1-dehydrogenase	P11413	59.1	6.44	5.84	3
M22	Glucose-6-phosphate 1-dehydrogenase	P11413	59.1	6.44	9.73	5
M23	Glucose-6-phosphate 1-dehydrogenase	P11413	59.1	6.44	12.65	6
M24	26S proteasome non-ATPase regulatory subunit 11	O00231	47.3	6.09	23.04	8
M25	Elongation factor Tu	P49411	49.5	7.26	10.62	4
M26	Elongation factor Tu	P49411	49.5	7.26	4.87	2
M27	Alpha-centractin	P61163	42.6	6.19	8.78	2
M28	Heterogeneous nuclear ribonucleoproteins C1/C2	P07910	33.7	4.95	16.01	4
M29	Heterogeneous nuclear ribonucleoproteins C1/C2	P07910	33 7	4.95	22.55	6
M30	Heterogeneous nuclear ribonucleoprotein H3	P31942	36.9	6.37	11.27	3
M31	Voltage-dependent anion-selective channel protein 1	P21796	30.6	8.63	19.5	4
M31	Esterase D	P10768	31.5	6.54	4.61	1
M32	Pyruvate kinase, isozymes M1/M2	P14618	57.8	7.95	33.21	14
M33	Pyruvate kinase, isozymes M1/M2	P14618	57.8	7.95	29.43	13
M34	Aspartate aminotransferase	P17174	46 1	6.57	8.74	3
M35	Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	37.4	8.97	9.92	3
M36	Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	37.4	8.97	9.92	3
M37	Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	37.4	8.97	7.37	2
M38	Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	37.4	8.97	7.37	2
M39	Phosphoglycerate mutase 1	P18669	28.7	6.75	8.3	2
M40	Superoxide dismutase [Mn]	P04179	24.7	8.35	10.36	2

[a]Spot ID corresponds to the numbers on gel images in Fig. 1 (Macula-SYPRO Ruby). [b]Accession No. corresponds to UniProtKB/Swiss-prot database [c]MW and pI are theoretical scores.

in the choroidal layer more specifically, sections were labeled with anti-PECAM1 antibody (Fig. 3H). PECAM1 is an adhesion molecule expressed at intercellular junctions between vascular endothelial cells [24] (Fig. 3J and 3K, green). Tropomyosin detected by TM311 (red) was expressed adjacent to PECAM1 (Fig. 3I and 3K).

Discussion

In this study, we identified and validated of proteins expressed in the macula and peripheral retina. The method, 2D gel electrophoresis, limits detection to proteins in aqueous soluble form. Nevertheless, a number



of proteins highly expressed in the macula were found.

One of the identified proteins was arrestin-C, which is known to be highly expressed in cone photoreceptors, which are densely located in the primate macula [6]. Previous SAGE analyses of the retina by Bowes Rickman *et al.* [3] have shown 1.4-fold higher transcription of arrestin-C in the macula compared to the peripheral

Fig. 2. Western blot of 5 proteins. Five micrograms of each sample from the peripheral retina and macula were loaded onto SDS-page gel (for γ-synuclein, 15 μg loading). After transferring to a PVDF membrane, the proteins were detected with antibodies specific to tropomyosin Br-1, Br-3 (A), γ-synuclein (B), E-FABP (C), arrestin-C (D), TM311 to tropomyosin (E), hnRNPs A2/B1 (F). Pieces of gel images (a–f, a'–f') correspond with the boxed areas in Fig. 1 (Peripheral-SYPRO Ruby and Macula-SYPRO Ruby). Lane P, peripheral retina; Lane M, macula.

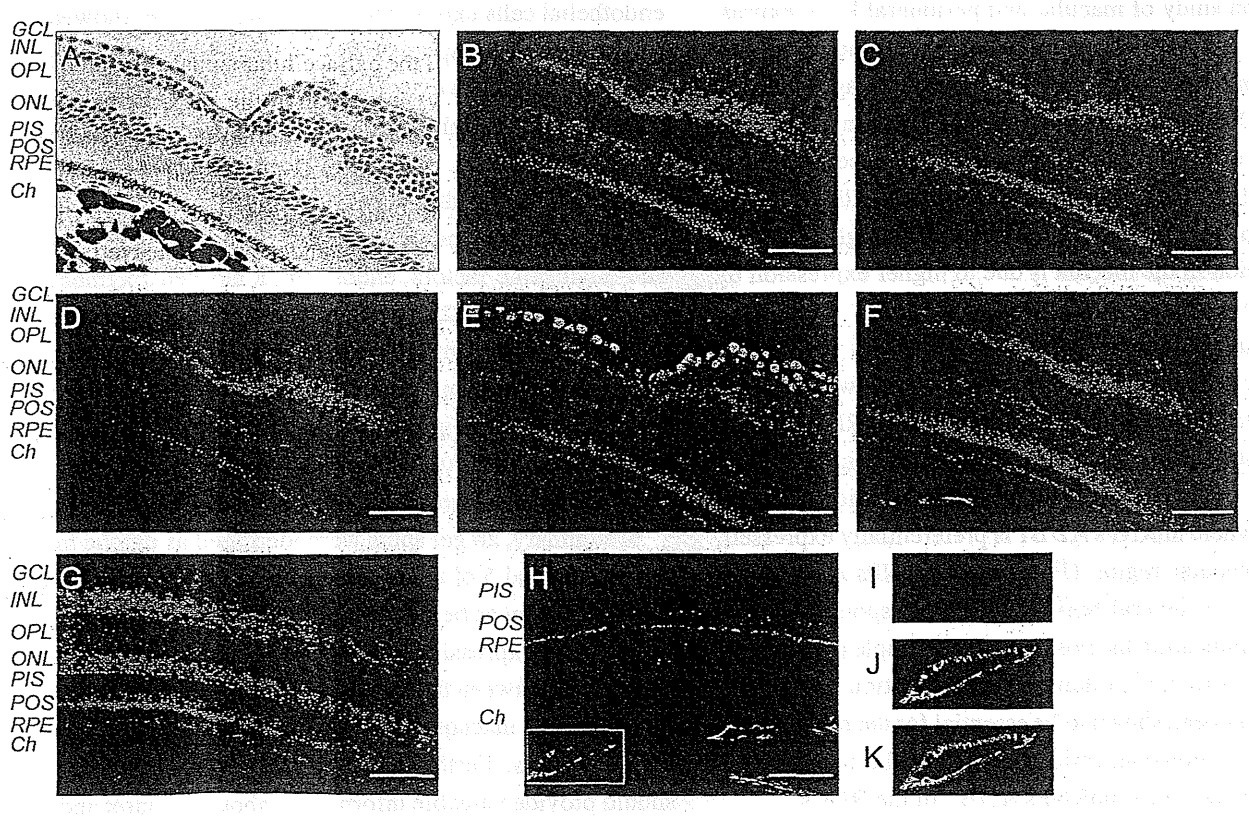


Fig. 3. Tissue localization of macula enriched proteins. Four-micrometer paraffin sections of monkey retina were stained with hematoxylin and eosin (A), other sections were labeled with antibodies specific to arrestin-C (B), tropomyosin Br-1, Br-3 (C), γ-synuclein (D), hnRNPs A2/B1 (E), TM311 to tropomyosin (F), and E-FABP (G) Tropomyosin was detected by TM311 in the choroidal layer (H). Boxed area in (H) is enlarged; labeled with antibodies specific to TM311 (red) (I), PECAM1 (green) (J), and merged (K). GCL, ganglion cell layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PIS, photoreceptor inner segment, POS, photoreceptor outer segment, RPE, retinal pigment epithelial, Ch, choroid (Bar; 50 μm)

retina in humans. Another protein that was identified in macular unique spots was 3'(2'),5'-bisphosphate nucleotidase 1 (Table 2, M3), which has also been identified by SAGE as being highly expressed in cone photoreceptors [3]. Identification of these cone photoreceptor rich proteins indicates that the present proteomic analysis was methodologically effective for identifying proteins richly expressed in the macula. It also suggests the higher protein level in the macula may be due to a higher density of specific cell types expressing specific proteins. Previous studies have shown arrestin-C expression in cones [33], γ-synuclein expression in RGCs [43], and E-FABP expression in Müller cells [21]. Although these proteins have been identified in not only specific cell types or compartments in the retina [1, 10, 15, 22, 50], the majority were localized in particular cell layers in the retina (Fig. 3B, 3D, and 3G). In a comparative transcription study of macular and peripheral RPE, expression of E-FABP was 6.3-fold higher in the peripheral RPE compared to macular RPE in middle-aged humans [48]. Our immunostaining showed predominant localization of E-FABP in the neural retina, except the photoreceptors (Fig. 3G), which is consistent with the results of Kingma *et al.* [21]. Our observation of higher E-FABP expression in the macula is due to higher expression by the neural retina, not the RPE.

Immunostaining revealed hnRNPs A2/B1 was present in every retinal nucleus layer (Fig. 3E). However, western blots showed higher expression of hnRNPs A2/B1 in the macula than in the peripheral retina (Fig. 2F). This may be explained by a higher concentration of RGC layers, where hnRNPs A2/B1 is preferentially expressed, in the macular region (Fig. 3E). hnRNPs A2/B1 was identified in several horizontal 2D gel spots (Fig. 1D, box. f') indicating the possibility of multiple phosphorylation sites for this protein. Phosphorylation of hnRNPs A2/B1 has been shown to be essential for the myelination of the axon-glia connection [51]. A similar myelination role is expected for hnRNPs A2/B1 in the RGCs.

In this study, two types of antibodies for tropomyosin isoforms were used. The brain-type isoform of tropomyosin detected by TMBr-3 antibody was not differentially expressed between the macula and peripheral retina (Fig. 2A), however tropomyosin detected by TM311 antibody showed remarkably higher expression in the macula (Fig. 2E). The difference in the expression level of the TM311-detected isoforms of tropomyosin resulted in an additional spot in the macula 2D gel which did not react with the TMBr-3 antibody. The TMBr-3-detected isoform is expressed in all regions of the brain [12, 41] and also in the outer plexiform layer and photoreceptor inner segments of the retina (Fig. 3C). This is in contrast to arrestin-C expression, which is limited to the photoreceptor outer segment (Fig. 3B). This is the first report to localize the brain-type isoform of tropomyosin in photoreceptors.

TM311 detected isoforms were localized to vascular endothelial cells compared to the localization of PECAM1 in choroid layer (Fig. 3H). Abundant expression of tropomyosin 1 α chain in the macula may arise from the higher capillary density in the choroidal layer of the fovea [30]. An earlier study, using human umbilical vein endothelial cells exposed to hydrogen peroxide, showed an increase in phosphorylation of tropomyosin through the activation of the extracellular signal-regulated kinase (ERK) pathway. Inhibition of the ERK pathway results in disruption of the endothelial layer and a two-fold increase in transendothelial permeability [17]. Tropomyosin has been previously described to interact with two anti-angiogenic factors, endostatin, a cleaved fragment of collagen XVIII [23], and high molecular weight kininogen [52]. Both proteins exhibit anti-angiogenic effect by binding to tropomyosin. Thus, tropomyosin may play an inhibitory role in increasing permeability or angiogenesis in the macula. Angiogenesis is a pathological finding often observed in the advanced stage of AMD [13].

In summary, 26 gel spots were identified as unique to the macula and 5 of these proteins were also confirmed by western blot as being richly expressed in the macula. Differential expression is likely due to morphological differences between the macula and the peripheral retina. The retina of macaque monkeys is almost identical to that of humans. Further understanding of these proteins should provide valuable information about the onset and progression of macular diseases in humans.

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Chapter 9
Suppression of Drusen Formation by Compstatin, a Peptide Inhibitor of Complement C3 activation, on Cynomolgus Monkey with Early-Onset Macular Degeneration

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Abstract For the past 10 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 months 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 sub-retinal space. The progressive impairment of the RPE and damage to Bruch's membrane and choriocapillaris results in retinal atrophy and photoreceptor dysfunction.

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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. 2006). 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 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 also identified in drusen. Additional proteins such as crystallins, EEFMP1, and amyloid-beta have been 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. 2005a, b). El-Mofty et al. (1978) 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. 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. 1996a, b; Suzuki et al. 2003). This single affected monkey has been bred to a large pedigree of more than 300 monkeys (Fig. 1). Drusen are observed in the macula as early as 2 years after birth, and the number increase and spread toward the peripheral retina throughout life (Figs. 2–3). Histological abnormalities of the retina and abnormal electroretinogram (ERG) were observed in sever case showing physiological dysfunction of the macula.

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. 2005a, b; 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.

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,

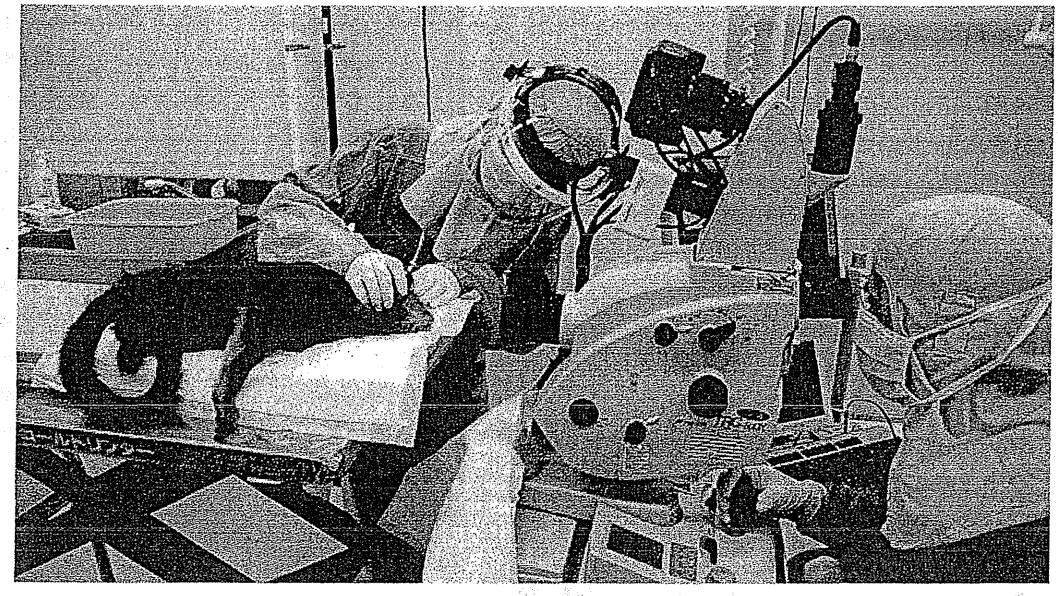

Fig. 1 Fundus photography of affected monkey at TPRC

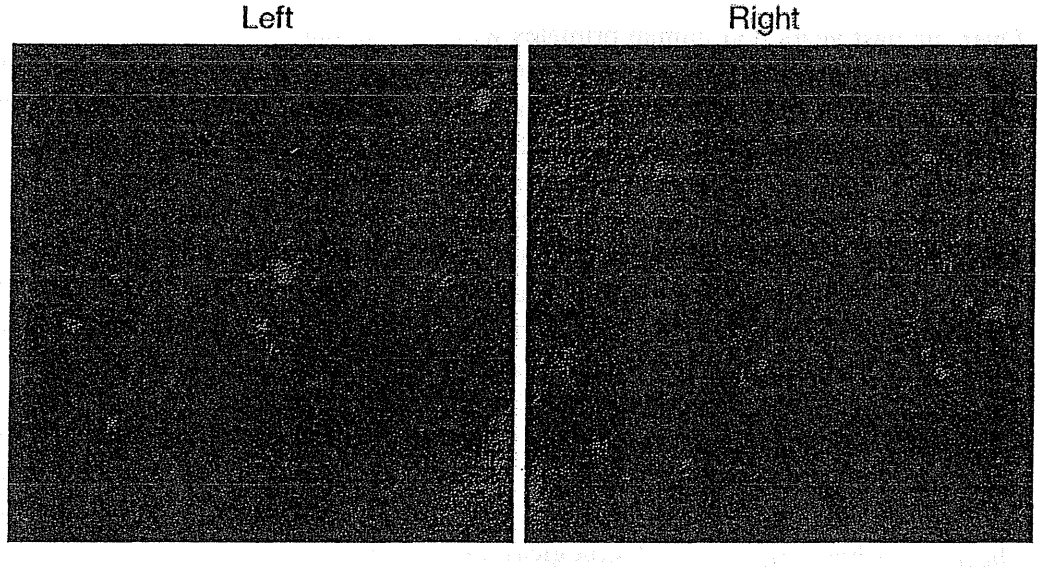

Fig. 2 Fundus photograph of affected monkey showing accumulation of drusen in macula of both eyes

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,

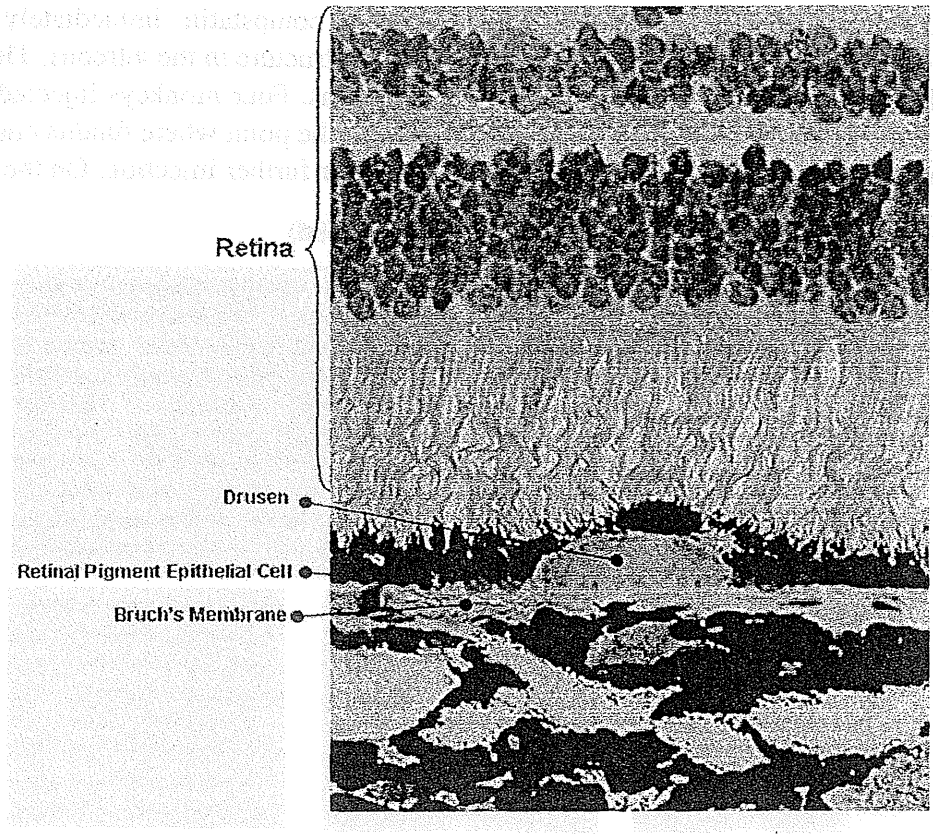

Fig. 3 Retinal histological section of affected monkey showing the accumulation of drusen

α1-antitrypsin, and apolipoprotein E), major histocompatibility complex class II antigens, and HLA-DR antigens (Umeda et al. 2005a, b). 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.

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 et al. 2006) was intravitreally injected into eight affected monkeys at different dose and intervals. Four affected monkeys were injected at 1 mg dose at 1 month interval while other four affected monkeys at 50 μg dose at 1 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 months. Four monkeys injected with 1 mg for 3 months developed significant opacity to the point where fundus observation was impossible. These monkeys were halted for further injection. On the other

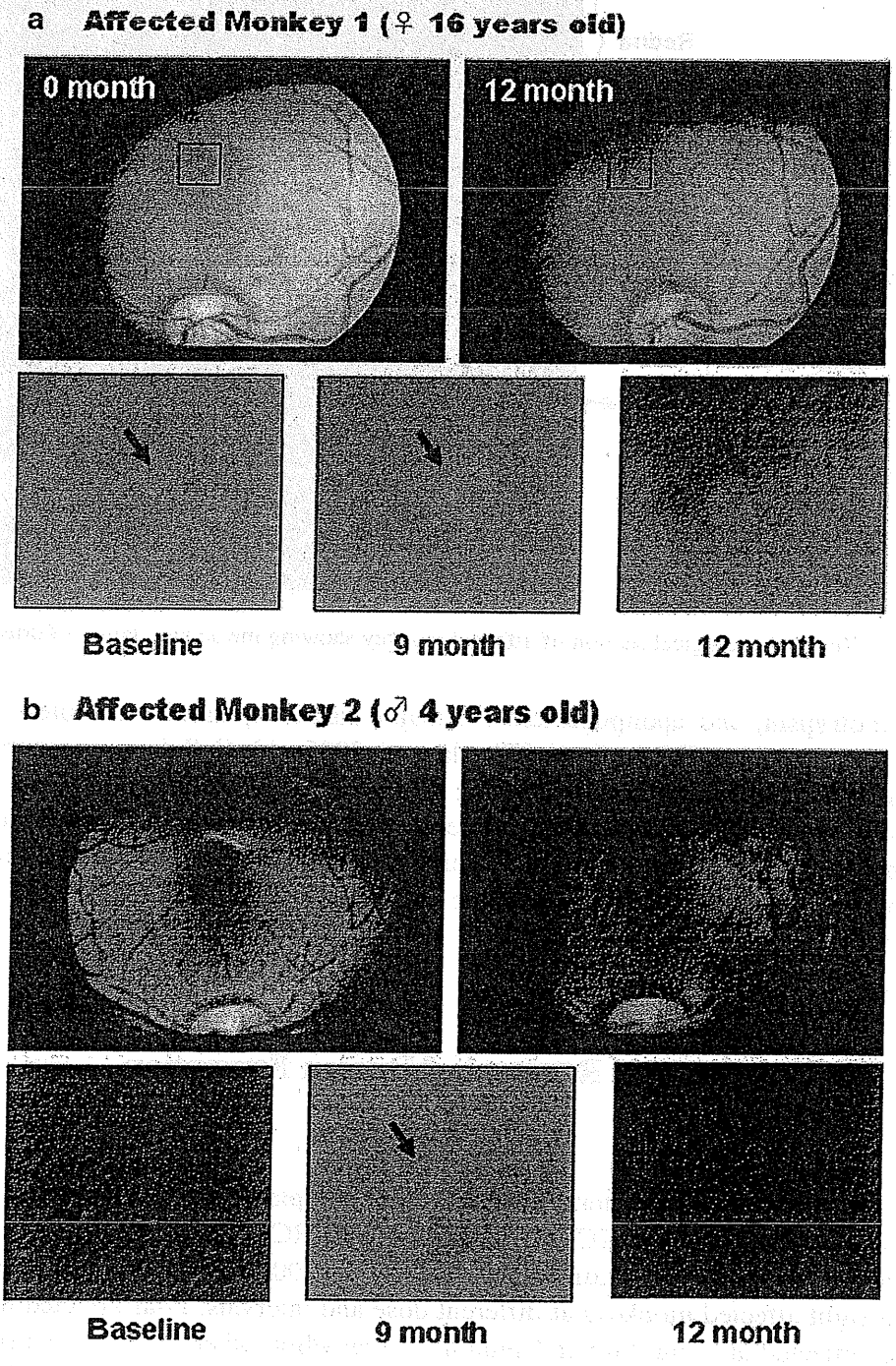

Fig. 4 Suppression and reversal of drusen formation after 9 months of intravitreal injection of 50 μl compstatin at 1 week interval

hand, vitreous of four monkeys with 50 μg dose were clear within 2 days. After 6 months of injection, we noticed diffusion of drusen in the macula and by 9 months partial disappearance of drusen was observed in all four 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.

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7 補体と疾患

7-6 眼科と補体

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はじめに

ヒトは情報の約8割を視覚情報に依存しており、生涯を通じて視機能を維持することは生活の質(QOL)を維持することに直結する重要な課題である。眼は外界に直接開かれていることから、感染や傷害からこの器官を守る必要があり、自然免疫系の重要な一角である補体が重要な役割を果たしている。眼は免疫特権部位(immune privileged site)と呼ばれる臓器に分類され、通常の免疫炎症反応が起こると透明性を維持できなくなるため、炎症反応を最小限に抑え、眼の透明性と微細構造を保護する特性を備えている[1]。

補体は角膜や硝子体の炎症に関与し、さらに最近の研究から、加齢黄斑変性や緑内障などの有病率の高い眼科疾患の発症にも関与することが明らかにされてきた。特に補体の遺伝子多型が加齢黄斑変性の発症リスクを高めることが統計学的に証明され、眼球内の補体を抑制するための薬が複数の製薬会社で研究されている。本章では眼における補体の関与について、特に加齢黄斑変性を中心に、最近の知見を紹介する。

1. 眼の基本的構造と機能

眼球はほぼ球形であり、前方の角膜から後極の強膜までの距離は新生児で約17mm、成人では約24mmである。角膜、前房、虹彩などから構成される前眼部、水晶体、硝子体などの中間透光体、そして網膜、脈絡膜、視神経乳頭から構成される後眼部に分けられる(図1)。光は透明な角膜で屈折し、虹彩によって光量が調整されながら眼の中に取り込まれる。毛様体の筋肉によって水晶体の厚みが変化することによって光はさらに屈折変化を起こして、無色・透明なゲル状の硝子体を通過し、網膜に結像する。網膜のほぼ中央に位置する黄斑の中心には視細胞が集中する中心窩が存在し、この高密度な視細胞の存在によって、一部の霊長類や鳥類には高い解像度の中心視力が確保された。視細胞で受容した光刺激は電気信号に変換されて、視神経、視交叉、視索を経て、後頭葉の視覚中枢に伝達され、映像として認知される。視神経は直径約3mmの神経の束であり、眼球を出ると有髄神経線維となって周囲からのノイズを遮断している。角膜から網膜までを保護する最外層の強膜は太さの不均一な膠原線維を中心とした細胞外基質で構成されている。

2. 眼における補体の存在

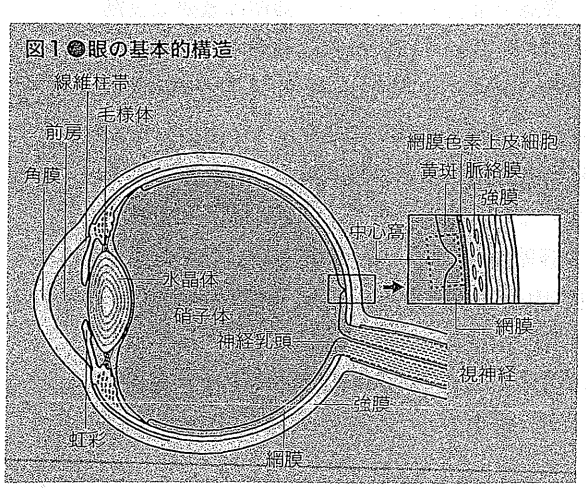
ヒトの主要な情報源である視覚情報を生涯にわたって受け取るために、眼は外界からの様々な危険に対して保護機能が用意されている。角膜から網膜までを光が通過するためには、規則的な分子・細胞の配列を乱すような炎症反応は大きな障害となる。眼の保護だけでなく、免疫特権部位としての機能を維持するためにも補体は重要な役割を担っている。これまでの研究報告によると、正常な眼球においてもiC3bやMACの存在が確認されており、補体は持続的に機能しているが、その活性は膜結合型補体制御因子によって厳密に制御されている[2]。角膜、房水、涙液、そして網膜では古典経路と副経路と、これを制御するためのC1阻害因子(C1-INH)、DAF、MCP、CD59、I因子、H因子などの存在が確認されている[3]。iC3bとそのレセプターは免疫特権部位を維持するために直接関与している[4]。また、これらの補

体制御因子に対する抗体を投与すると重篤な炎症反応が生じることが報告されている。

3. 角膜疾患と補体

角膜は眼瞼や結膜とともに直接外界に露出しており、様々な微生物による感染や抗原によるアレルギーなどが発生する。角膜は無血管の透明な組織で、表裏はともに滑らかな非球面を構成しており、中央部の厚さは約0.5mmである。角膜の屈折率は1.37とほぼ水と等しく、光は角膜で強く屈折して眼球内に入る。角膜の表面は油層と水層からなる涙液層に覆われており、角膜上皮細胞を保護している。角膜は上皮、Bowman膜、実質、Descemet膜、内皮の5層に分かれている。上皮は5、6層の上皮細胞から構成されており、基底部の細胞は一定の間隔で分裂し、上方へ移動して、最終的には表層から脱落する。

角膜では、古典経路と副経路の補体因子(C1、C2、C3、C4、C5、C6、C7、プロペルジン、B因子)が確認されている。ヒトドナーの角膜にリポ多糖(LPS)、リビトールテイコイン酸、塩酸、水酸化ナトリウムなどを少量垂らし、アナフィラトキシン(C3a、C4a、C5a)の濃度を測定した実験によると、いずれの場合もアナフィラトキシンが検出されたが、いずれについてもMACは観察されなかった。コブラ毒因子(cobra venom factor)によってC3因子を阻害されたマウスの角膜に緑膿菌(*Pseudomonas aeruginosa*)を感染させた実験では、炎症反応は観察されていない。さらに、遺伝子改変によってC5因子遺伝子が欠損したマウスにおいても炎症反応は観察されていない。炎症反応の行き過ぎから角膜を保護するために、MCP、DAF、Crry、CD59などの膜結合型補制御蛋白質が高発現しているが、感染するバクテリアによってはホスホリパーゼなどの酵素を産生し、角膜表面からDAFやCD59が脱落する危険性がある。角膜における可能性のある炎症抑制薬として、アナフィラトキシンをターゲットにした抑制薬が有効と考えられている。



4. ぶどう膜炎と補体

ぶどう膜は虹彩、毛様体、脈絡膜から構成されており(**図1**)、これらの組織はメラニン色素と血管が非常に豊富で、その色調がぶどうに似ていることからぶどう膜と総称されている。ぶどう膜炎は炎症の発生部位により、4つの病型(前部、中間部、後部、汎ぶどう膜炎)に分類されている。ぶどう膜炎は発生部位における炎症から白内障、緑内障、嚢胞様黄斑浮腫などへと周辺へ病気が進行する場合もある。日本人のぶどう膜炎の原因としては、ベーチェット病(Behçet disease)が約10～20%、サルコイドーシスが約10～20%、フォークト・小柳・原田病(Vogt-Koyanagi-Harada disease)が約5～10%、そして原因不明が約40～50%を占める。

この疾患と補体との関連についてはまだ十分な研究がなされていないが、ぶどう膜炎患者の房水中のC3bやC4bが検出されている。ぶどう膜炎の根本的な原因はまだ明らかにされていないが、モデル動物を用いた研究によって自己免疫疾患であることが明らかにされている。実験的に作製された自己免疫性ぶどう膜炎(experimental autoimmune anterior uveitis：EAAU)のモデル動物においてもC3bの上昇が観察されている。この動物モデルはウシのメラニンに結合した抗原を完全フロイトアジュバントを用いてマウスや

ラットの大腿部皮下に注射し、12～14日後に虹彩、毛様体の炎症から始まり、16～19日後には網膜の炎症を誘発する方法である。C3bとCR3との相互作用が発症機序に深く関係していると考えられており、抗CR3抗体を静注することによってモデル動物の発症を予防できることが報告されている。補体の活性化は複数のサイトカイン、ケモカイン、接着分子の発現を上昇させ、同時にIFN-γ、IL-10、IP-10、ICAM-1、LECAM-1などが減少することから、ぶどう膜炎の発症には補体制御蛋白質一群の関与が考えられる。

5. 糖尿病網膜症と補体

糖尿病網膜症は糖尿病の3大合併症の1つで、中途失明原因1位の眼科疾患である。糖尿病を主原因として、網膜毛細血管の増殖とこれを構成する周辺細胞の脱落および内皮細胞の浸透性の増加によって、未熟な血管から血液が漏れてくることにより、広範囲の視神経障害が発生する。この疾患においても補体の関与が報告されている。MACやC3dの免疫染色は陽性であるが、古典経路やレクチン経路に関係するC1qやC4の免疫染色は確認されていないことから、副経路の関与が示唆されている。しかしながら、糖尿病患者の硝子体中にB因子、C3、C4b、C9などが検出されていることから、発症晩期においては古典経路の活性化も発生していることになる。

6. 視神経シナプスの構築と緑内障における補体

緑内障は、視細胞が光から変換した電気信号を脳に伝える視神経が障害され、高眼圧あるいは正常眼圧において、視神経乳頭の陥凹、視神経の委縮、特徴的な視野狭窄を来たす疾患として定義されている。眼圧を十分に下げることによって進行を防止あるいは遅延させることできることが多いが、根源的な治療法ではなく、その発症原因の解明が急務である。近年行われた日本緑内障学会と岐阜県多治見市による疫学調査(多治見スタディー)によると、40歳以上における有病率は5%で、70歳以上の高齢者になると15%に上昇すると報告されている。また角膜と虹彩の間(隅角)が開放状態の開放隅角緑内障患者において、その9割が正常眼圧であったと報告されている。初期には自覚症状がほとんど現れないので、潜在的な患者数は多いと予測されている。

緑内障の障害は神経節細胞の萎縮が主原因であるが、近年の研究によって視神経シナプスの形成から緑内障による視神経の消失においても補体が関係していることが明らかになってきた。神経節細胞はその発生過程で網膜から視覚情報を処理する外側膝状体(lateral geniculate nucleus：LGN)へ投射し、シナプスを形成するが、生後数週間は必要なシナプスを選択的に残して残りは消失する。C1qあるいはC3の欠損マウスではこの選択が阻害され、精巧に機能する視神経が形成されないことが明らかにされた[5]。また、緑内障のマウスモデルを用いて外側膝状体の切片を観察するとC1qの発現が抑制されていることが報告されている。

7. 加齢黄斑変性と補体

加齢黄斑変性は中心視野が障害される加齢性の眼科疾患で、厚生労働省によって難治性疾患に指定されている。米国では失明率が最も高い疾患であり、わが国でも生活の欧米化や急速な高齢化、そして診断法の確立によって患者数は増加している。黄斑の中心に位置する中心窩には色を感じる錐体細胞が高い密度で存在し、これによってヒトは高い解像度の中心視力を得ている。中心窩では大量の視覚情報が通過・処理されており、この生理機能を維持するために、血管の豊富な脈絡膜と神経網膜との間で盛んに酸素、栄養素、老廃物の交換が行われている。網膜と脈絡膜の境界には網膜色素上皮細胞が存在し、分子輸送に加えて視細胞の貪食作用や各種因子の分泌機能などを併せもって網膜の恒常性を維持している。この細胞層が老化によって機能低下すると、細胞内で十分に

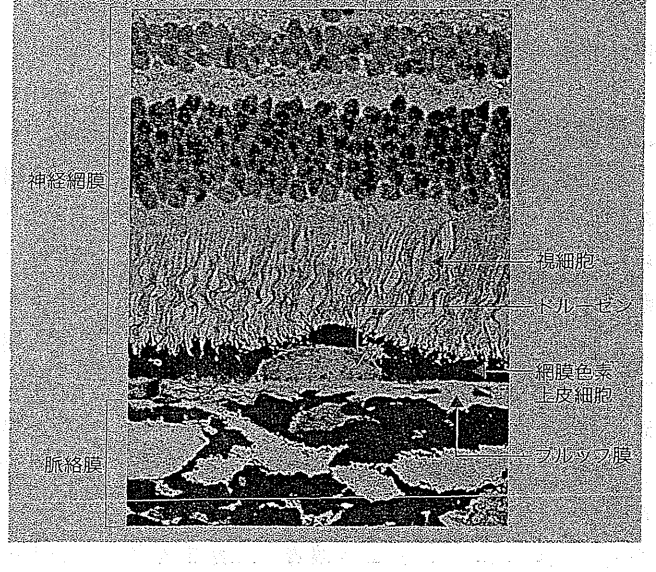
分解・処理できず、これが細胞外へ放出されて、ドルーゼンとして網膜色素上皮細胞とブルッフ膜の間に蓄積する(図2)。

ドルーゼンの蓄積が黄斑を中心に広範囲に及ぶと、これに接する網膜色素上皮細胞は徐々に萎縮し、黄斑部の視細胞も障害されて萎縮型加齢黄斑変性となる。これは脈絡膜から視細胞に向かって黄斑部で血管新生が起こる滲出型加齢黄斑変性と区別される。萎縮型は白人での頻度が高く、滲出型は日本人に多いことが知られている。ドルーゼンの生成メカニズムはまだ十分に解明されていないが、遺伝子多型、視細胞を保護する不飽和脂質(DHA)の光酸化分子に対する自己抗体、アミロイドβの蓄積によるマクロファージの誘導、ウイルス感染による炎症など複数の原因が考えられている。この結果、ドルーゼンや網膜色素上皮細胞に補体の活性化が確認されており、患者の網膜切片の免疫染色によって補体関連分子の陽性反応が観察されている。萎縮型の患者の一部は滲出型へ移行することが知られているが、その詳細なメカニズムは不明のままである。加齢黄斑変性のリスク因子として、遺伝子、加齢に加えて、喫煙、肥満、青色光などが知られている。

加齢黄斑変性と補体との関連が注目されたのは、患者の網膜切片において、ドルーゼンや網膜色素上皮細胞に補体の活性因子や抑制因子が免疫染色によって確認されてからである[6]。さらに、その数年後にはアメリカ人の患者を対象に行われた全ゲノム相関解析(genome wide association study：GWAS)によって、H因子(CFH)、B因子(CFB)、C2、C3などの遺伝子多型(single nucleotide polymorphism：SNP)が発症と相関することが明らかにされた[7]。この中でも、特にH因子のY402H(rs1061170)の遺伝子多型は白人やヨーロッパ系インド人において多くの患者について相関したのに対して、日本人や中国人ではY402Hの相関は観察されず、I62V(rs800292)が一部の患者で相関する程度であった[8]。今後のアジア人におけるH因子の遺伝子多型が注目される。Y402HはH因子の反復配列(short consensus repeats：SCRs)の7番目にあり、C3b、C反応性蛋白(C-reactive protein)、グリコサミノグリカンとの結合部位に位置し、副経路の制御に影響すると考えられる。H因子のノックアウトマウス(*cfh-/-*)では視細胞の障害、網膜におけるC3の蓄積、ブルッフ膜の菲薄化が観察されている。

以上の事実から、補体の活性化を抑制することによって加齢黄斑変性を治療することが考えられ、多くの補体抑制薬が臨床試験でその薬効が評価されている。黄斑は一部の霊長類と鳥類にしか存在しないために、厳密には一般的な実験動物(マウス、ラット、モルモット)では黄斑に関係する実験はできない。筆者らは独立行政法人医薬基盤研究所霊長類医科学研究センターとの共同研究によって、若年で患者と同成分のドルーゼンを生成する遺伝性の黄斑変性カニクイザルを解析している。この疾患サルにおいて、ヒトと同様にドルーゼンや網膜色素上皮細胞において補体の活性化が観察されている[9]。筆者らは補体を抑制することによってドルーゼンの生成を抑制あるいは消滅できるか、C3b(Compstatin、John Lambris)

図2 神経網膜と脈絡膜の間に蓄積するドルーゼン



およびC5b（AcPepA、岡田秀親）抑制薬の効果を研究中である。先行しているCompstatinについては、実験に用いた4頭全頭について、一部のドルーゼンについて消失していく様子が観察された（**図3**）。AcPepAについても同様な研究結果が期待されている。

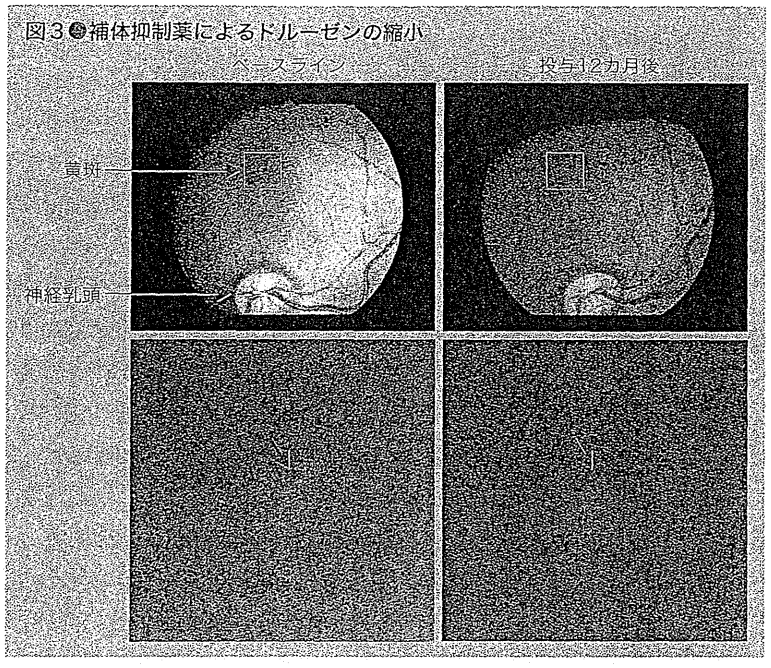


図3 補体抑制薬によるドルーゼンの縮小

おわりに

補体は眼を感染から守る防御機構であると同時に視神経の構築にもたずさわる重要な役割を果たしている。本項で紹介したように、補体は多くの眼科疾患に関与しており、診断への応用のほか、補体の活性化を制御することによる予防法や治療法が期待されている。すでに他の疾患用に開発された補体抑制薬が眼科疾患への応用を目的として、数多くの臨床試験が進行中である。しかしながら、眼の特性からこれらのすべての薬が利用できるわけではなく、また患者の負担を軽減するためにも、その投与方法の工夫が必要と考えられる。情報の約8割を担う視機能の維持は生活の質と直結しており、眼における補体の機能をさらに深く研究する必要を訴えたい。

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