

TABLE 3. (continued).

Gene	Exon No.	Length (bp)	Name	Forward Primer	Name	Reverse Primer	Size (bp)
<i>ELOVL4</i>	4	387	MP4aF	CCCTCTTAGAAGATTCTGACTTA	MP4aR	ACACTCCACTGGTTGCCAT	249
			MP4bF	ATGAACAGCCTCAGCAGGA	MP4bR	GCAAAAAGTTTTGGATGGTTA	316
	5	123	MP5F	GGAGCCAATATCAACATCTTCA	MP5R	TGCTTGAGCTTGAAACAGTTAAG	248
	6	120	MP6F	GCAAACAGCAATGCTAATFCA	MP6R	GAAATACTGCAACATGGCATG	250
	7	120	MP7F	CAGCTAGGCAATATTTATCAGCA	MP7R	CAGGGATTGGACTTTATTCCA	279
	8	120	MP8F	ATATCCAAAGTAGTGGTGACAA	P8R	TTCTGGTTAAACTAATACGCTAACA	235
	9	124	MP9F	TGCAAACAGAATCTGCCAGTA	MP9R	TTTGGCTTGTAAGACCAGAA	265
	10	196	MP10F	CTTACCAAGCCAACTGCTAACTA	MP10R	AACAACTCCCCTCTTCTCAATAG	289
	11	162	MP11F	AAAGCATAGAACTCCAAATGCA	MP11R	AGGTAACAATATTCTTTGGCTGACT	281
	1	100	MP1F	CCGGCGTTAGAGGTGTTTC	MP1R	GAGACGAGGGCTCGGTGAC	281
	2	188	MP2aF	TTGAGACATCTTGATTCTAGAAAAG	MP2aR	AAGTTAAGCAAAAACCATCCCA	252
		MP2bF	CTGGGTCCAAAGTGGATGAA	MP2bR	AGCTAACAGTTATGTCTGGGTACAA	213	
3	81	MP3F	GCAATTGGAATGCATGACA	MP3R	TTCACAGATTGGGGCCTATA	304	
4	172	MP4aF	AAATGATTCCATGCCTTGATACA	MP4aR	AACGCAAGCAGTATATTCCTGA	330	
		MP4b	TGCTGTTTATAACAGGCTTTCC	MP4bR	CTGATTGCTTTCCACTGAACA	271	
5	128	MP5F	ATCTCGGTGGCTTACTGCTTA	MP5R	AATAAGTCGGCTGGAGTCAACT	356	
6	276	MP6aF	TTGGCCCTGTGATGCTATG	MP6aR	TTAGGCTTTTGTATGTCCGAA	247	
		MP6bF	CTCTAATGCCTACGCAATCAG	MP6bR	GGGAGTTTTTCTCACTGTCA	242	
<i>TIMP3</i>	1	121	MP1F	AACTTTTGAGAGGCGAGCA	MP1R	CCTAAGCAGCGCTGCAGTC	233
	2	83	MP2F	TGAGATGCTGTTCTGATGTG	MP2R	GGCTGGTCTTAGACACACA	266
	3	112	MP3F	AGCACTGGGATTATGGATCATA	MP3R	ACATTTGGTGAAGTCACTACTCA	267
	4	122	MP4F	TGGGCTAAGTGGCAACATAGTA	MP4R	GTTTCTAGGGCTGCAAGTCA	274
	5	198	MP5F	TACCATGGCAGATTCCATCA	MP5R	AGTTACTGTCCGAGGGAAGCT	306

* Exon 2, 7, and 10 of the *VMD2* gene were screened for sequence variants only by direct sequencing.

Linkage Analysis

Linkage analysis was performed on DNA from 19 affected and 7 unaffected members of the pedigree. Individuals used for the analysis are indicated by asterisks in Figure 1. Human microsatellite markers linked to human macular degeneration loci were analyzed with monkey genomic DNA used as the template. Details of microsatellite markers and their primer sequences were obtained from the genome database. Microsatellite marker analysis was performed by two methods: Markers linked to candidate gene loci and included in a linkage mapping set (ver. 2.5MD10; Applied Biosystems, Inc. [ABI], Foster City, CA) were analyzed on the a DNA sequencer (model 3100; ABI) with fluorescence-labeled primers. Additional microsatellite markers were analyzed by ³²P dCTP incorporation into the amplified product.³¹ Two-point linkage analysis was performed between the disease locus and microsatellite markers with the MLINK program of the LINKAGE package, as described elsewhere.^{32,33} Linkage was assessed under the conditions of autosomal dominant inheritance of the disease trait with a frequency of 0.001 for the disease-causing allele, by using the affecteds-only model, as published earlier.³⁴ Linkage analysis was performed assuming equal frequencies for marker alleles. Haplotypes were constructed with genotypes of microsatellite markers according to their order on human chromosomes.

RESULTS

Clinical and Histologic Findings

Fundus photographs and FA of a 14-year-old female affected monkey (Fig. 1, monkey A) are shown in Figure 2. Fine, yellowish white dots were observed in the maculae (Figs. 2a-d), scattered in the peripheral retina along blood vessels in this monkey (Figs. 2a, 2b). However, in most cases, the locations of the lesions fell within the region centered on the fovea centralis with the same diameter as one optic disc. FA showed hyperfluorescence corresponding to these dots, except foveola (Figs. 2e, 2f). No abnormalities were found in the optic disc, retinal blood vessels, or choroidal vasculatures in any eyes examined. The amplitude and peak latency of both dark- and light-adapted ERG showed no alteration compared with normal

control eyes, indicating that global rod or cone degeneration was absent. Histologic studies demonstrated that there were various-sized drusen, weakly stained by PAS (light purple), between the RPE and choriocapillaris in the macular region (Figs. 3a, 3b, asterisk). These drusen were strongly reactive with antibodies against complement C5 (Figs. 3c, 3d). This finding was consistent with the property of drusen reported in patients with AMD.³⁵ Accumulation of lipofuscin in RPE cells was also obvious by PAS (Figs. 3a, 3b, deep purple, arrows).

Mutation Analysis of the *ABCA4*, *VMD2*, *EFEMP1*, *TIMP3*, and *ELOVL4* Genes

To evaluate the involvement of the *ABCA4*, *VMD2*, *EFEMP1*, *TIMP3*, and *ELOVL4* genes in disease, we first determined the genomic sequence and the complete cDNA sequence of the orthologous genes in the monkey. Subsequently, these genes were screened for sequence variants in affected and unaffected monkeys in the pedigree, in addition to unrelated, unaffected animals by SSCP, or by DHPLC for the *ABCA4* gene, analysis and direct sequencing.

***ABCA4*.** The monkey *ABCA4* gene consists of 50 exons, with its translation stop codon in exon 50, similar to the human gene. The complete 6819-bp cDNA encodes a protein of 2273 amino acids. *ABCA4* is a member of the superfamily of ATP-binding cassette (ABC) transporters, which are associated with membranes and transport various molecules across extra- and intracellular membranes of all cell types. ABC genes typically encode four domains that include two conserved ATP-binding domains and two domains with multiple transmembrane segments. Comparative sequence analysis revealed that the monkey *ABCA4* protein was only 1.8% (41 amino acids) different from the human orthologue, whereas the sequence was identical in the two adenosine triphosphate (ATP)-binding domains. Five of the 41 nonconserved amino acids in the monkey protein (codons 223, 423, 1300, 1817, and 2255) involve polymorphisms in the human. Surprisingly, the Lys223Gln and Arg1300Gln changes reported to be associated with Stargardt disease in humans were observed in the homozygous state in

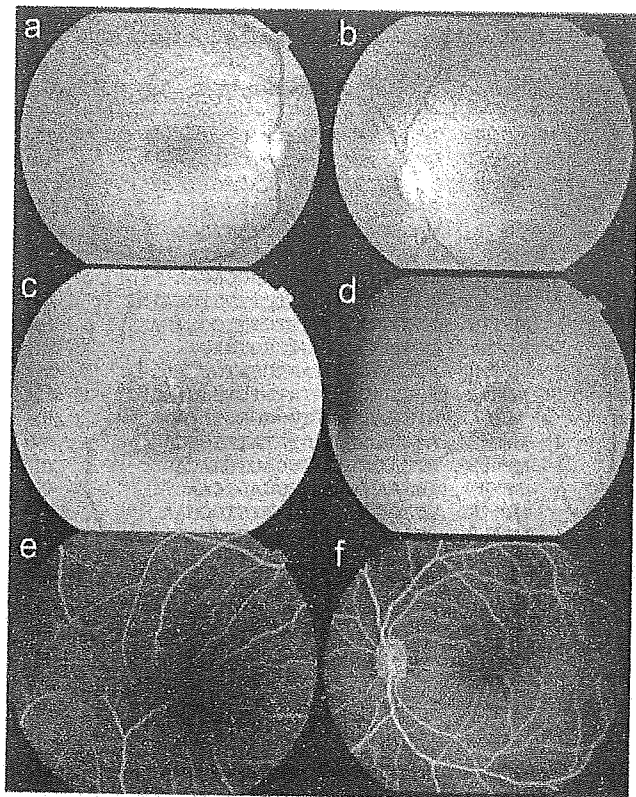


FIGURE 2. Fundus photographs and fluorescein angiogram (FA) of a 14-year-old female cynomolgus monkey (Fig. 1, monkey A) with macular degeneration, showing the right (a, c, e) and left (b, d, f) posterior poles. Fine grayish white or yellowish white dots were visible in the macula (a-d). The dots were observed in the peripheral retina along blood vessels in this monkey (a, b). These dots showed hyperfluorescence in FA except in the foveola (e, f). High-magnification of the macular region (c, d, e).

one normal control monkey (Fig. 1, monkey C). In addition, the mutation analysis revealed heterozygous amino acid changes at five positions—Leu424Val, Arg1017His, Val1114Ile, Ile1615Val, and Pro2238Gln—in both affected and normal monkeys. However, these missense variants did not segregate with the disease phenotype.

VMD2. The monkey *VMD2* gene consists of 11 exons, with its translation initiation codon in exon 2, as observed in its human orthologue. The complete cDNA was 2187 bp, encoding 585 amino acids. The *VMD2* gene encodes the bestrophin protein, which localizes to the basolateral plasma membrane of the RPE with the postulated function as an oligomeric chloride channel.^{36,37} The hydrophathy profile predicted that bestrophin contains four stretches of hydrophobic amino acids that function as transmembrane domains. Comparative sequence analysis demonstrated that monkey bestrophin had 19 amino acids different from its human homologue, and the four putative transmembrane domains are highly conserved. To date, 72 disease-associated nucleotide substitutions of the *VMD2* gene have been identified in patients with Best disease.^{3,7,26} The mutation analysis of the *VMD2* gene in the monkey pedigree detected six amino acid sequence variants. A polymorphism (Val/Ile) was detected at codon 275 in the fourth transmembrane domain, which has also been reported in humans.²⁶ Four polymorphisms (Tyr465His, Thr542Met, Glu557Gln, and Thr566Ala) were detected in exon 10. These changes did not segregate with the disease. In addition, one nonsense mutation at codon 582 (Glu→Stop) in exon 11 was detected in two

normal monkeys, whereas none of the examined six affected monkeys showed the change.

EFEMP1. The exon-intron gene structure of the monkey *EFEMP1* gene was also similar to the human *EFEMP1* gene. It was composed of 11 exons with its translation initiation codon in exon 2. The complete cDNA was 2034 bp, encoding 493 amino acids. Although the function of this gene remains unclear, this class of proteins is known to have characteristic sequence of repeated calcium-binding EGF-like domains.⁴ The monkey *EFEMP1* cDNA was found to have six EGF repeats. Four EGF repeats (numbers 2-5) are encoded by single exons (exons 5-8), one EGF repeat (number 1) is encoded by three exons (exons 2-4), and EGF repeat number 6 is encoded by two exons (exons 9, 10). This finding is in agreement with one of the two transcriptional variants with a distinct 5' untranslated region (UTR) described in its human homologue. Comparative sequence analysis demonstrated that the monkey *EFEMP1* has three amino acids different from that of the human, but the sequence in the entire region of six EGF repeats is completely conserved. In humans, a single mutation (Arg345Trp) that disrupts one of these domains is known to cause Malattia Leventinese.⁴ No amino acid-changing polymorphisms were found in all the monkeys tested. Three single nucleotide polymorphisms (SNPs), that did not alter the amino acid sequence, were detected in exons 4, 5, and 10.

TIMP3. The monkey *TIMP3* gene consisted of five exons, similar to its human orthologue. The complete cDNA was 1887 bp in length, encoding 211 amino acids. *TIMP3* is the third member of the tissue inhibitors of metalloproteinase family, a group of zinc-binding endopeptidases involved in the degradation of the extracellular matrix. *TIMP3* has 12 cysteines characteristic of the TIMP family, which are proposed to form intramolecular disulfide bonds and tertiary structure for the functional properties of the mature protein. The predicted amino acid sequence of the monkey *TIMP3* gene was identical with the human orthologue, including the 12 cysteine residues. Mutations in the *TIMP3* gene are known to cause Sorsby's fundus dystrophy.⁵ With a few exceptions,^{38,39} most previously described mutations disrupt the disulfide bonds by changing residues into cysteines, leading to misfolding of the protein.^{5,40} No coding sequence changes were detected in the *TIMP3* gene in monkeys by mutation screening.

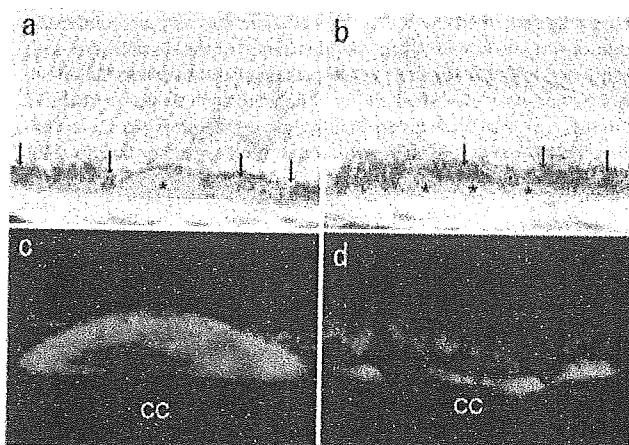


FIGURE 3. Drusen in the affected monkey retina. An affected 14-year-old male monkey (Fig. 1, monkey B). There were various-sized drusen, which were weakly stained by PAS (*), between the RPE and choriocapillaris (CC) (a, b). These drusen were strongly reactive with antibodies against complement C5 (green channel). Lipofuscin autofluorescence is shown (red) in the RPE (c, d). Accumulation of lipofuscin in RPE cells was also obvious by PAS (a, b, arrows).

TABLE 4. Two-Point Lod Scores between the Monkey Macular Degeneration Locus and Markers at the Human Macular Degeneration Loci

Markers	Distance from the Gene (CM)	Order on the Chromosome (M)	Lod Scores at θ									Exclusion ($Z = -2$)
			0	0.001	0.005	0.01	0.05	0.1	0.2	0.3	0.4	
<i>CORD8</i>		154.28										
<i>DIS431</i>	10.5	165	-ε	-2.116	-1.422	-1.128	-0.483	-0.248	-0.071	-0.01	0.006	0.001
<i>DIS2635</i>	0	154.28	-ε	-11.078	-7.598	-6.112	-2.773	-1.469	-0.392	0.019	0.119	0.075
<i>DIS2715</i>	-6.9	147.01	-ε	-7.7	-4.925	-3.747	-1.162	-0.232	0.388	0.464	0.299	0.03
<i>DIS498</i>	-10.6	144.94	-ε	-1.124	-0.439	-0.154	0.416	0.564	0.567	0.433	0.227	0.0001
<i>ABCA4</i>		94.1										
<i>DIS188</i>	-2.3	91.7	-ε	-6.139	-4.058	-3.175	-1.24	-0.541	-0.05	0.074	0.066	0.01
<i>DIS2849</i>	-1.2	92.9	-ε	-1.766	-1.075	-0.784	-0.166	0.032	0.133	0.119	0.067	
<i>DIS2868</i>	0.1	94	-ε	-14.824	-10.623	-8.809	-4.599	-2.846	-1.264	-0.522	-0.146	0.1
<i>STGD3</i>		80.5										
<i>D6S1662</i>	-2.67	77.83	-ε	-1.232	-0.544	-0.257	0.324	0.476	0.472	0.34	0.17	0.0
<i>D6S1048</i>	0.28	80.78	-ε	-0.063	0.614	0.889	1.38	1.416	1.172	0.79	0.362	0.0
<i>D6S1596</i>	7.1	87.6	-ε	-8.746	-5.965	-4.78	-2.138	-1.127	-0.319	-0.025	0.049	0.05
<i>D6S1609</i>	12.08	92.58	-ε	-7.326	-5.235	-4.34	-2.302	-1.475	-0.724	-0.349	0.131	0.05
<i>DHRD</i>		56.1										
<i>D2S2230</i>	3.9	60	-ε	-11.691	-8.209	-6.719	-3.349	-2.006	-0.842	-0.325	-0.084	0.1
<i>D2S378</i>	1.1	57.2	-ε	-9.268	-6.482	-5.29	-2.593	-1.517	-0.588	-0.186	-0.019	0.05
<i>ARMD1</i>		192.2										
<i>DIS384</i>	-2.11	190.09	-ε	-5.565	-3.486	-2.606	-0.696	-0.032	0.375	0.389	0.236	0.01
<i>DIS413</i>	2.1	194.1	-ε	-11.068	-7.59	-6.106	-2.784	-1.501	-0.46	-0.067	0.047	0.05
<i>DIS2622</i>	3.7	195.9	-ε	-1.961	-1.271	-0.982	-0.375	-0.185	-0.084	-0.066	-0.047	0.0
<i>VMD2</i>		61.5										
<i>D11S1993</i>	-2.3	59.2	-ε	-1.615	-0.925	-0.636	-0.032	0.151	0.224	0.181	0.1	0.0
<i>D11S4174</i>	1.4	62.9	-ε	-7.132	-5.026	-4.112	-1.979	-1.102	-0.368	-0.087	0.003	0.01
<i>D11S4076</i>	7.3	66.8	-ε	-5.617	-3.537	-2.656	-0.736	-0.061	0.364	0.385	0.231	0.01
<i>Rhodopsin</i>		130.6										
<i>D3S3515</i>	-4.01	126.59	-ε	-2.756	-1.379	-0.803	0.383	0.717	0.775	0.584	0.302	0.001
<i>D3S3720</i>	-2.8	127.8	-ε	-2.626	-1.247	-0.67	0.531	0.879	0.945	0.729	0.389	0.001
<i>D3S1269</i>	0.3	130.9	-ε	-11.566	-8.081	-6.588	-3.2	-1.846	-0.7	-0.238	-0.062	0.05
<i>Timp3</i>		31.5										
<i>D22S1162</i>	7.05	38.55	-ε	-3.587	-2.203	-1.619	-0.365	0.055	0.291	0.276	0.159	0.005
<i>D22S280</i>	0	31.5	-ε	-4.051	-2.664	-2.075	-0.785	-0.321	-0.002	0.065	0.044	0.01
<i>D22S273</i>	-1	30.5	-ε	-1.878	-1.187	-0.896	-0.278	-0.078	0.026	0.025	0.004	0.0
<i>CTRP5</i>		118.7										
<i>D11S4127</i>	-1.6	117.1	-ε	-0.771	-0.088	0.192	0.73	0.827	0.719	0.495	0.244	0.0
<i>D11S924</i>	0.2	118.9	-ε	-1.424	-0.736	-0.449	0.137	0.298	0.322	0.232	0.113	0.0
<i>D11S4129</i>	4.48	121.58	-ε	-9.057	-6.275	-5.089	-2.435	-1.41	-0.566	-0.214	-0.054	0.05
<i>STGD4</i>		26.1										
<i>D4S403</i>	0	26.1	-ε	-16.798	-11.919	-9.83	-5.081	-3.159	-1.445	-0.633	-0.206	0.1
<i>D4S391</i>	1.2	27.3	-ε	-3.615	-2.231	-1.647	-0.392	0.026	0.255	0.234	0.13	0.005
<i>CORD5</i>	(Interval)	64.5										
<i>D17S938</i>	0	64.5	-ε	-16.296	-11.422	-9.339	-4.638	-2.776	-1.176	-0.466	-0.125	0.1
<i>D17S796</i>	0	64.5	-ε	-3.594	-2.209	-1.624	-0.358	0.075	0.324	0.305	0.176	0.0
<i>MCDR1</i>	(Interval)	98.1										
<i>D6S434</i>	4.3	102.4	-ε	-4.496	-3.103	-2.507	-1.163	-0.632	-0.183	-0.005	0.043	0.0
<i>CORD9</i>	(Interval)	47.6										
<i>D8S1820</i>	0	47.6	-ε	-11.981	-8.501	-7.014	-3.65	-2.277	-1.002	-0.385	-0.092	0.1

ELOVL4. We have reported cloning and characterization of the *ELOVL4* gene in the cynomolgus monkey.⁴¹ Three mutations leading to truncation of the *ELOVL4* protein were reported in humans with Stargardt-like macular dystrophy^{23,42} (Karen G, et al. *IOVS* 2004;45:ARVO E-Abstract 1766). Mutation analysis of monkeys with macular degeneration did not detect any amino acid-altering sequence changes. Silent polymorphisms were observed in exons 1, 3, and 4 of the *ELOVL4* gene.

Linkage Analysis of Candidate Gene Loci

The methodology we used to screen for mutations in the candidate genes could miss disease-associated changes that may be present in the promoter or intronic regions; therefore, linkage analysis was performed to exclude the five genes further. Moreover, the macular degeneration phenotype in the

monkey pedigree could be caused by a single gene defect. In these cases, linkage analysis would be a comprehensive approach to confirm or exclude a particular gene locus. Microsatellite markers linked to the five candidate gene loci in addition to eight human macular degeneration loci—*ABCA4*, *VMD2*, *DHRD* (*EFEMP1*), *TIMP3*, *STGD3* (*ELOVL4*), Cone rod dystrophy-8 (*CORD8*), age-related macular degeneration 1 (*ARMD1*, gene Hemicentin1), rhodopsin, *STGD4*, North Carolina macular degeneration (*MCDR1*), *CORD9*, late-onset retinal degeneration (*CTRP5*), and *CORD5* loci—were analyzed to test for linkage with the macular degeneration in the monkey pedigree. None of the tested loci gave significant positive lod scores (Table 4). We also constructed haplotypes using the genotype data of markers at the 13 loci. This analysis further supported the exclusion of these loci from being among those that might harbor the gene associated with macular degeneration in these monkeys.

DISCUSSION

We report a detailed description of early-onset macular degeneration in cynomolgus monkeys and the exclusion of known genes responsible for macular degeneration in humans as a disease-associated gene in this animal model. Several forms of macular degeneration have been described in humans, including autosomal dominant, autosomal recessive, and X-linked modes of inheritance. The most common form of macular disease in humans is AMD. Major clinical characteristics of AMD are loss of central vision with RPE atrophy or exudation. The presence of subretinal deposits known as drusen is one of the early signs observed in AMD and several other macular degenerations. Recent studies suggest that the process of drusen formation includes inflammatory and immune-mediated events.³⁵ Immunohistochemical examinations have revealed that drusen contains activated complement factors. These molecules include C5, the cleavage product of C3 (C3b, iC3b, and C3dg), and the terminal complement complex C5b-9. Clinical and histologic studies of the affected monkeys showed the presence of drusen (Figs. 2, 3). Immunologic analysis demonstrated that drusen in monkeys had C5 as a component, suggesting that the nature of monkey drusen was similar to that reported in human AMD. At the same time, the onset of the disease in monkeys is at ~2 years of age; therefore, the monkey macular degeneration resembles early-onset human macular degeneration with drusen.

Comparison of the gene maps and chromosome painting data revealed a high degree of synteny and genome conservation between human and Macaque genomes.^{43,44} Amplification of cynomolgus monkey DNA with human microsatellite marker primers and sequence analysis revealed that not only the sequences flanking the microsatellite repeat regions but also the polymorphic nature of these repeats is conserved between human and monkey genomes (data not shown). Comparative studies on human and chimpanzee genomes have shown the same average heterozygosity at microsatellite marker loci and conserved genetic distance between markers.⁴⁵ Molecular cloning of monkey orthologues of the human *ABCA4*, *VMD2*, *EFEMP1*, *TIMP3*, and *ELOVL4* genes further demonstrated the high conservation between the human and macaque genomes not only in the organization of the gene structure, but also at the sequence level. Considering the high conservation between human and macaque genomes, human macular degeneration loci can be considered plausible candidates for identification of the gene associated with macular degeneration in the monkeys. We tested this hypothesis using microsatellite markers linked to human macular degeneration loci and successfully amplified microsatellites in the monkey DNA with human primers. However, we failed to establish linkage with the tested loci, and the subsequent haplotype analysis further confirmed this finding. Therefore, the macular degeneration locus in the monkey pedigree is not likely to be associated with the regions of the monkey genome that are syntenic to human genomic regions comprising the 13 macular disease loci tested. Mutation analysis of candidate genes also supported the exclusion of the *ABCA4*, *VMD2*, *EFEMP1*, *TIMP3*, and *ELOVL4* genes. The analyses detected five- and six-amino-acid substitutions in the *ABCA4* and *VMD2* genes, respectively. Some silent nucleotide substitutions or intronic sequence changes, such as small insertions/deletions, SNPs, and variations of short tandem repeats were observed in the *EFEMP1*, *TIMP3*, and *ELOVL4* genes. All these sequence variants did not segregate with the disease phenotype in the extended pedigree. Hence, these changes were interpreted as benign polymorphisms.

In the *ABCA4* sequence of a normal monkey, we found two amino acid replacements (K223Q and R1300Q) that are associated with Stargardt disease in humans. Because of the exten-

sive conservation between the monkey and human gene sequences, one would expect these amino acid changes to have similar disease-associated effects in monkeys. One explanation of this discrepancy could be that K223Q and R1300Q are not causing the disease phenotype in humans, but rather represent markers linked to disease-causing mutations somewhere else in the gene. Alternatively, the disease-causing effect of these amino acid changes on the function of the human *ABCA4* protein could be eliminated or compensated for by other differences in the monkey protein. Comparative analysis of the monkey and human genes may provide clues for understanding the molecular pathogenesis caused by *ABCA4* variation. In the *VMD2* gene sequence of normal monkeys, we found a nonsense mutation at codon 582. The change is located at the fourth residue from the C terminus. Bestrophin was shown to form oligomeric chloride channels in cell membranes.³⁷ The C-terminal cytosolic tail, encoded by exons 10 and 11, has been reported not to be essential for the protein's function. Moreover, although 72 nucleotide substitutions have been identified in Best disease to date,^{3,7,26} none of them is reported in exons 10 and 11. Hence, the deletion of four amino acids from the C-terminal end of the protein could be considered not to be associated with the disease.

In summary, we demonstrated that none of the 13 human macular degeneration loci tested were involved in causing the macular degeneration phenotype observed in the monkey pedigree. These results demonstrate the need for additional studies to identify the genetic locus associated with the phenotype in these monkeys and to understand the genetic defect underlying the disease. Identification of the gene responsible for this specific macular degeneration phenotype not only defines a new candidate locus for human macular degeneration, but also provides a primate animal model that can be extensively studied for elucidation of the mechanisms, diagnosis, prophylaxis, and treatment of macular degenerations, including AMD.

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Molecular composition of drusen and possible involvement of anti-retinal autoimmunity in two different forms of macular degeneration in cynomolgus monkey (*Macaca fascicularis*)

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ABSTRACT

We have previously reported a cynomolgus monkey (*Macaca fascicularis*) pedigree with early onset macular degeneration that develops drusen at 2 yr after birth (1). In this study, the molecular composition of drusen in monkeys affected with late onset and early onset macular degeneration was both characterized. Involvement of anti-retinal autoimmunity in the deposition of drusen and the pathogenesis of the disease was also evaluated. Funduscopic and histological examinations were performed on 278 adult monkeys (mean age=16.94 yr) for late onset macular degeneration. The molecular composition of drusen was analyzed by immunohistochemistry and/or direct proteome analysis using liquid chromatography tandem mass spectroscopy (LC-MS/MS). Anti-retinal autoantibodies in sera were screened in 20 affected and 10 age-matched control monkeys by Western blot techniques. Immunogenic molecules were identified by 2D electrophoresis and LC-MS/MS. Relative antibody titer against each antigen was determined by ELISA in sera from 42 affected (late onset) and 41 normal monkeys. Yellowish-white spots in the macular region were observed in 90 (32%) of the late onset monkeys that were examined. Histological examination demonstrated that drusen or degenerative retinal pigment epithelium (RPE) cells were associated with the pigmentary abnormalities. Drusen in both late and early onset monkeys showed immunoreactivities for apolipoprotein E, amyloid P component, complement component C5, the terminal C5b-9 complement complex, vitronectin, and membrane cofactor protein. LC-MS/MS analyses identified 60 proteins as constituents of drusen, including a number of common components in drusen of human age-related macular degeneration (AMD), such as annexins, crystallins, immunoglobulins, and complement

components. Half of the affected monkeys had single or multiple autoantibodies against 38, 40, 50, and 60 kDa retinal proteins. The reacting antigens of 38 and 40 kDa were identified as annexin II and μ -crystallin, respectively. Relative antibody titer against annexin II in affected monkeys was significantly higher than control animals ($P < 0.01$). Significant difference was not observed in antibody titer against μ -crystallin; however, several affected monkeys showed considerably elevated titer (360–610%) compared with the mean for unaffected animals. Monkey drusen both in late and early onset forms of macular degeneration had common components with drusen in human AMD patients, indicating that chronic inflammation mediated by complement activation might also be involved in the formation of drusen in these affected monkeys. The high prevalence of anti-retinal autoantibodies in sera from affected monkeys demonstrated an autoimmune aspect of the pathogenesis of the disease. Although further analyses are required to determine whether and how autoantibodies against annexin II or μ -crystallin relate to the pathogenesis of the disease, it could be hypothesized that immune responses directed against these antigens might trigger chronic activation of the complement cascade at the site of drusen formation.

Key words: liquid chromatography tandem mass spectroscopy

Age-related macular degeneration (AMD) is the most common cause of legal blindness in people over 60 yr of age and is estimated to affect millions of individuals in industrialized countries. Among people over 75 yr of age, mild or early forms occur in nearly 30% and the advanced form in ~7% of the population (2). Taking high levels of antioxidants and zinc are shown to reduce the risk of developing advanced form by the Age-Related Eye Disease Study (AREDS) (3). The AREDS formulation, while not a cure for AMD, may play a key role in helping people at high risk for developing advanced AMD keep their remaining vision. At present there is no fundamental cure for AMD, although some success in attenuating choroidal neovascularization has been obtained with surgical excision or photodynamic therapy.

Major clinical characteristics of AMD are loss of central vision with choroidal neovascularization and geographic atrophy, where atrophy occurs around the choriocapillaris with clear boundaries. The accumulation of debris-like material between the retinal pigment epithelium (RPE) and Bruch's membrane is observed to precede this exudation and atrophy. Although the most prominent lesion of AMD involves the RPE and Bruch's membrane, it is degeneration, dysfunction, and death of photoreceptors and its consequences that account for the vision loss. Very little is known about the pathophysiology of this disease process. The debris-like material, referred to as drusen, is regarded as a hallmark risk factor for developing AMD. The presence of numerous and/or confluent drusen in the macula is widely accepted as a sign of the early stage of AMD, whereas their composition and mechanism of formation remains controversial.

Drusen or drusen-like bodies have been reported in macaque monkeys since the 1970s (4). Aged monkeys spontaneously show macular degenerative changes, such as pigment mottling, hyper- or hypopigmentation, and drusen in the macula (5, 6). The late onset form of macular degeneration in these monkeys is consistent with the phenotype observed in the early stage of AMD. Thus, macaque monkeys have been suggested as an optimum animal model for AMD (7, 8). In addition, we have previously reported an early onset macular degeneration in a cynomolgus monkey pedigree maintained at Tsukuba Primate Center (9–11). For these monkeys,

the symptoms appear early in life around the age of 2 yr and progress slowly throughout life. The disease has been shown to have autosomal dominant inheritance (12). These two forms of macular degeneration, late onset and early onset, in monkeys could be extremely valuable models of the early stage of AMD, especially for elucidating the mechanism of drusen formation. However, the molecular properties of drusen observed in monkeys have not been described to date. Comparative studies of the molecular composition of drusen in monkeys and humans are required to establish these macular degeneration monkeys as AMD models.

Drusen composition and origin have been analyzed extensively in AMD. Various lipids, polysaccharides, and glycosaminoglycans have been identified as constituents (13). Recent immunohistochemical studies have revealed that drusen contains protein molecules that mediate inflammatory and immune processes (14, 15). These components include immunoglobulins, components of the complement pathway, modulators of 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, α 1-antitrypsin, and apolipoprotein E), major histocompatibility complex class II antigens, and HLA-DR antigens. Cellular components have also been identified in drusen, including RPE debris, lipofuscin, and melanin. These findings have led to the suggestion that immune complex-mediated inflammation damages RPE cells, while RPE cells respond by secreting proteins that modulate the immune response. Shedding or endocytosis of cell membranes of injured RPE is postulated to function as the core for these secreted components to accumulate and form extracellular deposits (13).

Furthermore, the codistribution of IgG and terminal complement complexes in drusen suggests an immune response directed against retinal antigens and immune complex formation (16). This hypothesis is supported by the presence of putative anti-retinal autoantibodies in the sera of patients with AMD (17, 18). Anti-retinal autoantibodies have previously been reported in a number of retinal diseases, including retinitis pigmentosa (19), paraneoplastic retinopathies (20), and retinal vasculitis (21). In addition, patients with membranoproliferative glomerulonephritis who suffer from glomerular injury caused by complement activation and immune complex deposition are known to develop drusen resembling those of AMD by ultrastructure and composition (22). To date, the role of anti-retinal autoantibodies in the pathogenesis of AMD has not been fully examined. It remains unknown whether the initiation of chronic inflammation and subsequent drusen formation require autoimmune-mediated events as a primary initiating factor. To clarify the role of autoimmunity in AMD, the antigens eliciting circulating anti-retinal autoantibodies need to be identified.

In this study, the molecular composition of drusen observed in late onset and early onset macular degeneration monkeys was investigated by immunohistochemistry and proteome analysis for comparison with drusen in AMD. Involvement of anti-retinal autoimmunity in late onset monkeys was subsequently examined. Anti-retinal autoantibodies in sera from affected monkeys were screened, and the immunogenic molecules eliciting these autoantibodies were determined by LC-MS/MS. Relative levels of autoantibodies against the identified antigens were determined in sera from affected and unaffected monkeys. Better understanding of the molecules involved in drusen composition and autoimmunity will improve evaluation of the macular degeneration monkeys as human AMD models. Furthermore, this information should also provide important clues to aid in the development of possible therapeutic reagents for prevention of drusen formation.

MATERIALS AND METHODS

Maintenance of monkeys

The cynomolgus monkey pedigree with early onset macular degeneration was reared in Tsukuba Primate Research Center, National Institute of Biomedical Innovation. All monkeys were treated in accordance with the rules for care and management of the Tsukuba Primate Center (11) under the Guiding Principles for Animal Experiments using Non-Human Primates formulated and enforced by the Primate Society of Japan (Primate Society of Japan, 1986). All experimental procedures were approved by the Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation. The monkeys used for studies of late onset macular degeneration were reared in large-scale breeding facilities in Manila, Philippines (Simian Conservation Breeding and Research Center, Inc.). 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.

Clinical studies

At the breeding facility of the Simian Conservation Breeding and Research Center, 278 female monkeys ranging from 13 to 25 yr old were examined. The mean age was 16.94 yr old, and the median age was 17 yr. The clinical examination was performed after tranquilization by intramuscular injection of 10 mg/kg ketamine-HCl (Ketalar-50; Sankyo, Tokyo). Approximately 20 min before examination of the ocular fundi, one drop of a mixture of 0.5% tropicamide and 0.5% phenylephrine hydrochloride (Mydrin-P; Santen Pharmaceutical, Oosaka, Japan) was instilled into each eye of each animal for dilation of the pupils. The cornea was kept moist with artificial tears. Fundus examination and fluorescein angiography (FA) were performed using a TRC50 fundus camera (Topcon, Tokyo, Japan). For FA, 0.5 ml of 1% fluorescein solution (Fluorescite; Alcon Japan, Tokyo, Japan) was intravenously injected.

Immunohistochemical studies of drusen components

Enucleated eyes were fixed in 10% neutralized and buffered formaldehyde solution at 4°C overnight and then dehydrated. The specimens were embedded in paraffin and sectioned to prepare serial sections of 4 µm thicknesses. The specimens were treated for antigen retrieval with 0.4 mg/ml proteinase K in phosphate buffered saline (PBS) for 5 min at room temperature or by autoclaving in Target Retrieval Solution (Dako, Carpinteria, CA) for 20 min at 121°C. Subsequently, the sections were blocked with 5% skim milk in PBS. The specimens were then reacted with primary antibodies diluted in PBS for 2 h at room temperature. Conditions for antigen retrieval and dilution of primary antibodies for each antigen are shown in [Table 1](#). After being washed, the sections were incubated with Alexa 488 conjugated goat anti-rabbit or mouse IgG (Molecular Probes, Eugene, OR) diluted 1:200 in PBS for fluorescent signal detection. The negative control stainings were performed with normal rabbit or mouse immunoglobulin fraction (Dako) instead of primary antibodies. After being processed, sections were examined using a confocal laser scanning microscope (Radiance 2100, Bio-Rad, Richmond, CA). Images were acquired with Lasersharp software. Double-labeled images were generated by the green channel for each antigen and red channel for autofluorescence emitted by lipofuscin pigment in the RPE.

Drusen isolation

After an eyeball was thawed on ice, the anterior segment was removed with a circumferential cut behind the limbus. The optic nerve was cut, and the posterior pole was laid open with longitudinal incisions leaving the macular region intact. The vitreous and neural retina were removed under a stereoscopic microscope (SMZ800, Nikon, Tokyo, Japan). The RPE was washed away from the interior surface of the globe with 100 mM ammonium bicarbonate buffer (pH 8.0). At magnifications between 20 and 30 diameters, drusen were scraped up with a tiny tungsten needle, the needlepoint of which was 1 μ m diameter (ST Japan, Tokyo, Japan), and transferred to ammonium bicarbonate buffer in tubes. Smaller drusen was collected by aspiration in the presence of the same buffer with a micro pipette (PrimeTech, Ibaraki, Japan) and a microinjector pump (Narishige, Tokyo, Japan). Isolated drusen were stored at -80°C until further analyses.

Direct proteome analysis of drusen components

Ten micrograms of isolated drusen suspended in ammonium bicarbonate buffer were dried and redissolved in 20 μ l of the same buffer. Cysteine was reduced by adding 20 μ l of 50 mM DTT and incubating for 1 h at 37°C. Subsequently, 20 μ l of 100 mM iodoacetamide were added and the alkylation continued 30 min at room temperature in the dark. The preparation was then digested with 1 μ g of trypsin at 37°C overnight. The resultant tryptic peptides were dried, resuspended in 40 μ l of aqueous 0.1% trifluoroacetic acid/10% acetonitrile, and analyzed by LC-MS/MS with a Paradigm system (Michrom Bioresources, Auburn, CA) and an ion trap mass spectrometer (LCQ DECA XP; Thermo Electron, Kanagawa, Japan). Peptides were separated on a Magic C18 column (200 μ m ID \times 5 cm, particle size 5 μ m, pore size 200 Å; Michrom Bioresources) by using aqueous formic acid/acetonitrile solvents, a flow rate of 3 μ l/min, and a gradient of 5–65% acetonitrile over 120 min. Protein identification from MS/MS spectra was performed using protein identification software (Bioworks 3.0, Thermo Electron) and National Center for Biotechnology Information protein sequence databases.

Screening for anti-retinal autoantibodies in affected monkey sera

The neural retina and choroid isolated from unaffected monkeys (4 yr old) were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.5% TritonX-100, 2% SDS, and protease inhibitors (Complete; Roche, Mannheim, Germany). After centrifugation at 16,000 g for 30 min at 4°C, the supernatant was collected. Fifteen micrograms of the extracted retinal proteins were mixed with sample buffer (Laemmli sample buffer; Bio-Rad), boiled for 3 min, and separated on 12.5% gel by SDS-PAGE. After transfer to PVDF membranes, the blots were cut into strips by single lane width. The individual strip was blocked with 5% skim milk in PBS containing 0.05% Tween 20 and then reacted with serum from an affected or unaffected monkey diluted (1:1000) in 2% BSA-PBS-0.1% Tween. Sera collected from 20 affected and 10 age-matched control monkeys were used. After incubation for 1 h at room temperature, the strips were washed four times with PBS-0.2% Tween and reacted with peroxidase-conjugated rabbit anti-human Ig (A+G+M) antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted (1:50,000) with 5% skim milk-PBS-0.1% Tween for 30 min at room temperature. After five washes, the strips were incubated with chemiluminescent substrate (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce, Rockford, IL). The resultant signals were detected and captured with Lumi-Imager F1 (Roche).

Identification of retinal autoantigens

Proteins were extracted from neural retina and choroid isolated from unaffected monkeys. Subsequently, the total protein solution was precipitated by changing solvent composition in a step-wise fashion such that a set of seven protein fractions was produced. These procedures were carried out using 2-D Fractionation kit (Amersham Biosciences, Buckinghamshire, UK). Eight micrograms of protein from each fraction were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with sera as described above. The protein fraction that reacted most intensively was dialyzed against 7 M urea/2 M thiourea at 4°C overnight. To the dialyzed protein solution was then added 4× sample buffer containing 200 mM DTT, 16% CHAPS, 0.8% carrier ampholytes. The samples were separated by 2-D electrophoresis. One hundred micrograms protein were loaded on immobilized pH gradient (IPG) strips (pH 3–10, 4–7, 7 cm; Bio-Rad) by in-gel rehydration at 20°C overnight. For the first dimension, isoelectric focusing (IEF) was performed with initial voltage 250 V for 15 min and then increased to 4,000 V for 1 h and held until 20,000 Vhr was reached. Immediately after IEF, 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 reducing 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 then electrophoresed for the second-dimension by SDS-PAGE. After transfer to PVDF membranes, immunoblotting with sera was performed as described above. The image of chemiluminescent signals was captured and merged with that of protein spots visualized by SYPRO Ruby (Bio-Rad), and the spots corresponding to the immunoreactivity were excised. The excised gel pieces were washed with 100 mM ammonium bicarbonate and then with acetonitrile. After the washing steps, gel pieces were completely dried for the reduction-alkylation step. The supernatant was removed, and the washing procedure was repeated three times. Finally, gel pieces were again completely dried before tryptic digestion and swelled in a solution of trypsin (12.5 ng/μl; Promega, Madison, WI) in 50 mM ammonium bicarbonate. The digestion was performed for 16 h at 37°C, and the extraction step was performed with 5% formic acid in 50% acetonitrile. The extracted peptides were pooled and dried. After being resuspended in 40 μl of aqueous 0.1% trifluoroacetic acid/10% acetonitrile, the samples were analyzed by LC-MS/MS as described above.

Expression and purification of recombinant proteins

The open reading frames of human annexin II and μ-crystallin were amplified by PCR from cDNA mixture synthesized from kidney, brain, liver, placenta, and lung (5'-RACE Ready cDNA; Clontech, Palo Alto, CA). Sense primer 5'-ATGTCTACTGTTACGAAATCCTG-3' and antisense primer 5'-TCAGTCATCTCCACCACACAG for annexin II, and sense primer 5'-ATGAGCCGGGTACCAGC-3' and antisense primer 5'-TTATTTACCAGATGACCAGGAATC-3' for μ-crystallin were used for amplification. The amplified products were subcloned into plasmid vectors (pTrc-His A; Invitrogen, Carlsbad, CA) with an N-terminal 6×His tag. The construct was transformed into *E. coli* (TOP10 cells; Invitrogen), and expression was induced with isopropyl-β-thiogalactoside. Bacteria were then lysed in buffer containing 8 M urea, 0.5 M NaCl, and 20 mM sodium phosphate (pH 7.4). Recombinant proteins were purified using affinity columns charged with Ni²⁺ ions (HiTrap Chelating HP; Amersham Biosciences), with a final elution using the same buffer with lowered pH (3.5).

ELISA for autoantibody titer

The purified recombinant protein was diluted (0.5 µg/ml) with sodium bicarbonate buffer (pH 9.6), and immobilized in 96-well immunoplates (Nalge Nunc, Rochester, NY). After being washed with 0.05% Tween 20 in PBS, the sample wells were blocked with sodium bicarbonate buffer containing 3% BSA for 2 h at room temperature. The sample wells were washed before the addition of sera diluted (1:50) with 1% BSA-PBS-Tween 0.05%. Sera collected from 42 affected and 41 age-matched control monkeys were used. After incubation for 2 h at room temperature, the plates were washed and reacted with peroxidase-conjugated rabbit anti-human Ig(A+G+M) antibodies (Jackson ImmunoResearch Laboratories) diluted (1:50,000) with 1% BSA-PBS-Tween 0.05% for 30 min at room temperature. After the final wash, 3,3',5,5'-tetramethylbenzidine substrate (Bio-Rad) was added to each well and incubated for color development. The reaction was stopped by adding 1 N HCl, and the absorbance at 450 nm was read.

Expression of annexin II in the retina

Protein extracts were prepared separately from the whole retina, neurosensory retina, and choroid including the RPE, which were isolated from unaffected monkeys, and also from cultured human primary RPE cells. The samples were applied to SDS-PAGE, transferred to membrane, and then immunoreacted with mouse anti-annexin II monoclonal antibody (Zymed Laboratories, South San Francisco, CA). Protein extract from Madin-Darby canine kidney (MDCK) cells, which are known to express annexin II abundantly, was used for positive control.

RESULTS

Clinical and histological findings of late onset macular degeneration monkeys

The fundus oculi of 278 aged monkeys (mean age: 16.94 yr) were funduscopically examined 3 times from 2001 to 2004. The fundus appearance typical of a monkey with late onset macular degeneration is shown in [Fig. 1A](#). Fine yellowish-white dots are observed in the macula. In the most cases, the locations of the lesions fell within the region centered on the fovea centralis within a diameter equal to one optic disc. These pigmentary abnormalities could be observed in 32% of the population. Of the 278 animals, 67.6% had normal macula with no detectable pigmentary abnormalities, 10.8% were diagnosed as a mild grade with fewer than 5 yellowish-white spots, 11.2% as a moderate grade with 5 to 20 spots, and 10.4% as a severe grade with more than 20 spots ([Table 2](#)). The most severe 12 cases were further examined by FA. FA of the same monkey is shown in [Fig. 1B](#). Hyperfluorescein dots could be observed corresponding to the spots in fundus photograph. Neither choroidal neovascularization nor disciform scarring was observed in any of the animals examined. No abnormalities were found in the optic disc or blood vessels. Histological studies were performed on 23 monkeys diagnosed as severe, including the 12 animals examined by FA. Drusen in the foveal or parafoveal region could be detected in eight monkeys unilaterally. The fundus and FA photographs of a typical monkey retina with drusen are shown in [Fig. 1C](#) and [D](#)). Hyperfluorescent dots had the same distribution as yellowish-white spots in the fundus photograph. In these eyes, various sized drusen accumulated between the RPE and choriocapillaris in the macular region ([Fig. 1E](#)). Drusen that had an eosinophilic inclusion could be observed (indicated by an asterisk in [Fig. 1F](#)). This spherical structure could be considered to originate from injured RPE cells, because it showed equivalent

autofluorescence to that emitted by lipofuscin granules in the RPE cells (Fig. 1G). Photoreceptor inner and outer segments appeared largely normal. In 15 of the 23 monkeys for which the eyes were examined histologically, including the monkey shown in Fig. 1A and B, drusen were not observed, but vacuolation and hyper- or hypopigmentation of the RPE cells could be observed corresponding to the yellowish-white spots in the fundus photographs (indicated by arrows in Fig. 1H). The vacuolated cells could be considered as aging, lipid-laden RPE cells.

Immunohistochemical and direct proteome analysis of monkey drusen

The protein components of drusen in monkeys were investigated by immunohistochemical methods. In addition to the eight monkeys affected with the late onset macular degeneration, which were histologically confirmed to have drusen, two affected monkeys from the pedigree with early onset macular degeneration were examined. Clinical and histological findings for drusen in early onset macular degeneration were described previously (1). Serial sections of the affected retinas with drusen were incubated with antibodies directed against proteins known to be present in drusen in AMD (14) (Table 1). All drusen in both late onset and early onset macular degeneration were heterogeneously bound by antibodies directed against apolipoprotein E (Fig. 2A and B), amyloid P component (Fig. 2C and D), complement component C5 (Fig. 2E and F), the terminal C5b-9 complement complex (Fig. 2G and H), and fluid phase inhibitor of complement cascade, vitronectin (Fig. 2I and J). The membrane-associated inhibitor of complement activation, membrane cofactor protein, was localized in membranous forms along the boundaries between drusen and RPE (Fig. 2K and L). These results indicated that chronic inflammation mediated by complement activation is also involved in the formation of drusen in monkey macular degeneration.

Subsequently, the molecular composition of drusen was further analyzed by direct proteome analysis using mass spectrometry. Drusen were isolated from the contralateral eyes of the four monkeys that were histologically confirmed to have drusen. The FA photograph of a monkey retina used in this experiment is shown in Fig. 3A. A number of drusen showing hyperfluorescence could be observed in the parafoveal region (indicated by a rectangle). After the posterior globe was laid open and the vitreous, neural retina, and RPE removed, drusen could be observed attached to the surface of Bruch's membrane at magnifications between 20 and 30 diameters under a stereoscopic microscope (Fig. 3B, white materials in a circle). Drusen were isolated with a tiny needle or a micropipette and transferred into ammonium bicarbonate buffer (Fig. 3C, arrows). The obtained protein yield was between 10 and 20 μg per preparation. The isolated drusen (10 μg) were digested with trypsin and analyzed by LC-MS/MS. As a result, we identified 60 proteins from three separate preparations and analyses (Table 3). Twenty of the identified proteins had been previously found to be components of drusen in AMD (indicated by bold letters in Table 3) (23). These proteins included annexin V, clusterin, crystallins, and immunoglobulins, in addition to the components identified by immunohistochemical studies, such as apolipoprotein E, complement components, and vitronectin. Additionally, seven proteins represented superfamilies in which other family members were known constituents of drusen in AMD, such as collagens, hemoglobins, histones, immunoglobulins, and tubulins (indicated by italic letters in Table 3). Therefore, one-half of the identified proteins in monkey drusen were identical to, or related to, known components of drusen from human AMD.

Autoimmunity against retinal proteins in late onset monkeys

The evidence of chronic complement activation at the site of drusen formation suggested that immune complex formation might be taking place via an immune response directed against retinal antigens. To evaluate the involvement of anti-retinal autoimmunity, sera from monkeys affected with late onset macular degeneration were immunoreacted with membrane blots of retinal proteins separated by SDS-PAGE. Sera collected from 20 affected animals and 10 age-matched control monkeys were used. Half of the sera from affected monkeys showed single or doublet reacting bands against 38, 40, 50, and 60 kDa proteins by Western blotting. Sera from the other affected monkeys, as well as the 10 unaffected animals, showed little or no reaction. To identify these four antigens, immunoblotting combined with 2-D electrophoresis was performed. After retinal protein extract was fractionated by stepwise precipitations, the fraction containing the highest concentration of the antigens of interest was selected by Western blotting. Subsequently, the selected fraction was separated on 2-D electrophoresis. An image of protein spots visualized by SYPRO Ruby is shown in [Fig. 4A](#). After transfer to PVDF membranes, the blot was reacted with sera containing autoantibodies. An image of chemiluminescent signals obtained by immunoreaction with the serum from the same monkey in [Fig. 1C](#) is shown in [Fig. 4B](#). Three immunoreactive spots were detected in a row at approximate size of 38 kDa. The images of protein spots and chemiluminescent signals were merged, and the corresponding protein spots were excised (indicated by circles in [Fig. 4A](#)). The excised protein spots were subjected to in-gel digestion with trypsin and were analyzed by LC-MS/MS. As a result, the proteins were identified as annexin II. Chemiluminescent signals obtained by immunoreaction with anti-annexin II monoclonal antibodies completely matched with those with the serum ([Fig. 4C](#)). By the same procedure, the 40 kDa antigen was found to be μ -crystallin, but the 50 and 60 kDa proteins could not be identified.

Evaluation of autoantibody by ELISA using recombinant antigens

Relative antibody titers against annexin II or μ -crystallin in sera collected from 42 affected monkeys with late onset macular degeneration and 41 age-matched control animals were determined by ELISA. The purified recombinant annexin II could be observed on SDS-PAGE gel at ~41 kDa ([Fig. 4D](#), lane 1). The recombinant proteins were confirmed to react both with anti-annexin II monoclonal antibodies (lane 2) and with autoantibodies in the sera (lane 3). Immunoreactivity against μ -crystallin was also confirmed by the same procedure (data not shown). These recombinant proteins were immobilized in 96-well plates for ELISA. Relative antibody titer against annexin II in affected monkeys was significantly higher than in control animals ($P < 0.01$; [Fig. 4E](#)). Seven affected monkeys showed more than twice the mean titer of the control group. On the other hand, relative antibody titer against μ -crystallin did not show significant difference between affected and unaffected monkeys. However, several affected monkeys showed considerably elevated titer (360–610%; [Fig. 4F](#)).

Expression of annexin II in monkey retina

The localization of annexin II in the retina was determined by Western blotting. Annexin II was present in protein extract from whole retina or choroid, but was most abundant in cultured human RPE cells ([Fig. 5A](#)). The result indicates that annexin II is highly expressed in the RPE cells both in vivo and in vitro. Immunohistochemical analyses failed to detect annexin II in the retinal cross sections but demonstrated remarkable expression in cultured RPE cells ([Fig. 5B](#)).

DISCUSSION

AMD is the leading cause of blindness in individuals over the age of 60 in industrialized countries. Limited access to human retinal tissues and the lack of good animal models in species with a well-developed macula make this disease difficult to study. Previous attempts to simulate AMD in experimental animals such as rodents through high-fat diets and phototoxicity (24, 25), senescence acceleration (26), or candidate gene manipulation (27–29) have not fully replicated the clinical and histological features of the disease. On the other hand, Macaque monkeys have been known to develop macular degenerative changes with age, including pigment mottling, hyperpigmentation, or hypopigmentation with drusen, consistent with the phenotype observed in the early stage of AMD (4–8). We have recently reported a monkey pedigree with early onset macular degeneration where drusen are observed <2 yr after birth (1). A well-developed macula is found only in primates and birds, thus making a primate model of particular value in elucidating the etiology and the mechanism underlying the disease. Such a value would also be an important bioresource to test new diagnostic techniques and potential therapeutic strategies for the prevention of this disease. However, previous characterization of monkey macular degeneration has not extended beyond clinical and histological studies. Here, we compare the molecular composition of drusen from monkeys with late onset and early onset macular degeneration with human drusen. The investigation extended the hypothesis of the involvement of anti-retinal autoimmunity in the etiology of AMD by identification of an autoantigen expressed in RPE cells.

The study was initiated from clinical observation of late onset macular degeneration in cynomolgus monkeys. A total of 278 aged animals were examined, and 32% of the population showed drusen-like spots in the macular region (Table 2). These affected monkeys were further classified into two clinical entities by histological studies. One was characterized by the formation of drusen (Fig. 1C–E) and the other by degenerative changes in RPE cells such as hyperpigmentation, hypopigmentation, and vacuolation (Fig. 1A, B, and H). These vacuolated lipid-laden RPE cells were observed as pigmentary abnormalities in fundus photographs, and window defects by these cells led to drusen-like hyperfluorescence in FA, making true drusen and lipid-laden RPE cells indistinguishable. However, the lipid-laden RPE cells were mostly individual solitary cells and not likely to represent the larger bodies such as those in Fig. 1C and D. This type resembles the “non-geographic atrophy” reported in the Chesapeake Bay Waterman Study (30) or the “pigmentary abnormality” of the International Classification and Grading System for Age-related Maculopathy and Age-related Macular Degeneration (31). In none of the affected monkeys examined have we observed choroidal neovascularization, disciform scarring, geographic atrophy, or other advanced pathological changes characteristic of later stage AMD. We have concluded the diagnosis of late onset macular degeneration monkeys as macular degeneration by drusen formation or RPE atrophy leading to the abnormal fundus appearances with pigmentary changes. Further examinations are required to determine the prevalence of drusen by histology in late onset macular degeneration monkeys.

Proteome analyses and immunohistochemical study of drusen composition demonstrated that monkey drusen had a number of protein components in common with drusen in human AMD (Fig. 2; Table 3) (23), including annexins, crystallins, immunoglobulins, apolipoprotein E, complement components, clusterin, and vitronectin. Similarities in the molecular composition of drusen suggested chronic inflammation mediated by complement activation driving drusen biogenesis as a common mechanism for both late onset and early onset macular degeneration in

monkeys. In addition, oxidative stress reactants calreticulin and ceruloplasmin were identified as drusen components by proteome analysis (Table 3). Calreticulin is a stress induced molecular chaperone protein of the endoplasmic reticulum. The protein also affects intracellular Ca^{2+} homeostasis and can modulate oxidative stress by blocking Ca^{2+} disturbance in the RPE cells (32, 33). On the other hand, ceruloplasmin is a ferroxidase, converting the hydroxyl-radical producing ferrous (Fe^{2+}) iron to the safer ferric (Fe^{3+}) form. Increased expression of ceruloplasmin has been reported in the mouse retina after photo-oxidation (34) and also in sera from AMD patients (35). In addition to these oxidative stress reactants, we observed accumulation of secondary oxidative products, such as 8-hydroxy-deoxyguanosine (8-OHdG) and 4-hydroxy-2-nonenal (4-HNE), in drusen and the surrounding neural retina in affected monkeys (data not shown). These observations indicate that oxidative stress participates in the pathogenesis of late onset macular degeneration in monkeys, as has been suggested in AMD.

Immunohistochemical studies of drusen composition also demonstrated that monkey drusen of late onset and early onset macular degeneration monkeys contained common components with drusen in AMD (Fig. 2). Drusen of late onset monkeys are observed in animals ranging in age from 13 to 25 yr, while drusen in early onset monkeys appear around the age of 2 yr (1). Recently, Ambati et al. (36) reported knockout mice lacking monocyte chemoattractant protein-1 or its cognate gene C-C chemokine receptor developed cardinal features of AMD, including accumulation of lipofuscin in RPE, photoreceptor atrophy, and choroidal neovascularization. Complement and IgG deposition in the RPE and choroid accompanies senescence in this model. The authors suggest that impaired macrophage recruitment may allow accumulation of C5a and IgG, leading to abnormal complement activation. A similar genetic defect that promotes the initiation of local chronic inflammation could be the cause of degeneration in the early onset monkeys. Further study of the molecular properties of drusen and identification of the disease-causing gene may provide important clues to the common causal events that trigger abnormal complement activation and subsequent drusen formation in AMD.

Involvement of anti-retinal autoimmunity against annexin II and μ -crystallin for late onset macular degeneration monkeys was also described in this study (Fig. 4). A previous report localized annexin II to the basal plasma membrane of the RPE (23). Moreover, this report identified annexins (annexin I, II, V, and VI) as drusen components in AMD. Annexin V was also identified in this study by proteome analysis of drusen composition as shown in Table 3. A possible pathological pathway whereby autoimmunity against annexin II could contribute to drusen formation is the following: 1) anti-annexin II immunoglobulins bind to the basal plasma membrane of the RPE; 2) the inactive C1 serum protein interacts with the Fc portion of the immunoglobulin; 3) this leads to formation of the C5b9 membrane attack complex; and 4) causing damage to the RPE cells followed by shedding of the cell membranes in the sub-RPE space. Immune complex formation might continue in the resultant drusen cores leading to further development of drusen.

Alternatively, anti-annexin II autoantibodies might contribute to the pathogenesis of the disease by impairing the normal functions of the protein. Annexins are Ca^{2+} and phospholipid binding proteins containing the annexin repeat motif and have been shown to interact with various ligands both outside and inside the cells to play multiple biological roles including the control of inflammatory responses (37). Annexin I is known to function as an anti-inflammatory mediator because of its response to glucocorticoids (38) and its activities in several animal models of inflammation (39, 40). Furthermore, high levels of anti-annexin autoantibodies have also been

reported in sera from patients with the common chronic inflammatory disease rheumatoid arthritis (RA) (41, 42). Continuous production of annexin autoantibodies that accelerate the inhibition of anti-inflammatory activity is suspected of contributing for the pathogenesis of RA. Similar mechanisms might be involved in the induction and maintenance of chronic inflammation in late onset macular degeneration monkeys.

Translocation of different annexins from the plasma membranes of phagocytic cells to the maturing phagosome membranes is believed to be involved in phagocytosis (37, 43). RPE cells perform numerous tasks essential for visual function, such as recycling of 11-*cis*-retinal for rod opsin, forming a barrier between the neural retina and the choroid, providing nutrients to the photoreceptors, and phagocytosis of rod photoreceptor outer segments. Disturbance of this latter process is likely to cause accumulation of debris and lead to retinal degeneration. Previous reports have shown that phagocytosis by RPE can be inhibited by an antiserum to RPE cell plasma membrane (44). Autoantibodies against annexin II could be one such inhibitory factor and may contribute to the pathogenesis of disease in late onset monkeys.

Several affected monkeys showed considerably elevated antibody titer against μ -crystallin compared with the control group (Fig. 4F). Crystallins are proteins expressed in very high abundance in the lens that are critical to the refractivity and transparency of the organ. μ -Crystallin is a taxon specific crystallin first described as a lens protein in several Australian marsupials (45). It binds NADPH is related to enzymes involve in aminoacid metabolism and is also expressed in photoreceptors and RPE. Other crystallins are also known to be synthesized by both the neurosensory retina and RPE, possibly functioning as stress proteins. Recently, crystallins were described as among the common proteins of drusen in human AMD (23). Our observations confirm crystallins are present in monkey drusen (Table 3). It can be hypothesized that injured RPE cells shed their cell membranes with cytoplasmic μ -crystallin into sub-RPE space exposing as new autoantigens. However, in this study, μ -crystallin expression was limited in the neural retina of normal control monkey compared with RPE (data not shown). One explanation of this discrepancy is that RPE cells compromised by some physical or metabolic stress might newly express μ -crystallin; in that case, the appearance of anti- μ -crystallin autoantibodies may be considered as a secondary event after RPE cell injury caused by chronic complement attack.

It still remains unclear whether autoantibodies against annexin II or μ -crystallin are the initial cause of the disease. It is possible that autoimmunity against these proteins might be the most critical event in the retina because annexin II is a ubiquitous protein and μ -crystallin is also expressed in brain, muscle, and kidney (46). Detailed clinical information on immunity in individual monkeys is essential to determine the primary cause of this disease. Although further analyses are required to define the relationship between the autoantibodies and the pathogenesis of the disease, autoantigens identified in this study strongly suggest the involvement of anti-retinal autoimmunity in AMD. Defining the AMD-related autoantibodies may provide possible diagnostic tools for the early detection and management of AMD.

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