Table 4. Identified proteins between 35 and 44 kDa that were produced by BRL cells and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Protein name	Molecular mass (Da)	Amino acid sequence[a]				
Actin gamma	42,092	1 MEEEIAALVI	DNGSGMCKAG	FAGDDAPRAV	FPSIVGRPRH	QGVMVGMGQK
		51 DSYVGDEAQS	KRGILTLKYP	IEHGIVTNWD	DMEKIWHHTF	YNELRVAPEE
		101 HPVLLTEAPL	NPKANREKMT	QIMFETFNTP	AMYVAIQAVL	SLYASGRTTG
		151 IVMDSGDGVT	HTVPIYEGYA	LPHAILRLDL	AGRDLTDYLM	KILTERGYSF
		201 TTTAEREIVR	DIKEKLCYVA	LDFEQEMATA	ASSSSLEKSY	ELPDGQVITI
		251 GNERFRCPEA	LFQPSFLGME	SCGIHETTFN	SIMKCDVDIR	KDLYANTVLS
		301 GGTTMYPGIA	DRMQKEITAL	APSTMKIKII	APPERKYSVW	IGGSILASLS
		351 TFQQMWISKQ	EYDESGPSIV	HRKCF		
Acyl coenzyme A hydrolase	46,268	1 MEATLSLEPA	GRSCWDEPLS	ITVRGLVPEQ	PVTLRAALRD	EKGALFRARA
		51 LYRADAHGEL	DLARAPALGG	SFTGLEPMGL	IWAMEPERPF	WRLVKRDVQT
		101 PFVVELEVLD	GHEPDGGRLL	ARAVHERHFM	APGVRRVPVR	EGRVRATLFL
		151 PPEPGPFPGI	IDLFGVGGGL	LEYRASLLAG	KGFAVMALAY	YNYDDLPKTM
		201 ETMRIEYFEE	AVNYLRGHPE	VKGPGIGLLG	ISKGGELGLA	MASFLKGITA
		251 AVVINGSVAA	VGNTICYKDE	TIPPVTILRN	QVKMTKDGLK	DVVDALQSPL
		301 VEQKSFIPVE	RSDTTFLFLV	GQDDHNWKSE	FYANEISKRL	QAHGKEKPQI
		351 ICYPEAGHYI	EPPYFPLCSA	GMHLLVGANI	TFGGEPKPHS	VAQLDAWQQL
		401 QTFFHKQLGG	KSHGVSPKI			
Capping protein (actin filament)	39,311	1 MYTPIPQSGS	PFPASVQDPG	LHIWRVEKLK	PWPIARESHG	IFFSGDSYLV
		51 LHNGPEEASH	LHLWIGQQSS	RDEQGACAVL	AVHLNTLLGE	RPVQHRELQG
		101 NESDLFMSYF	PRGLKYREGG	GRVGISQDNL	RATPAAIRKL	YQVKGKKNIR
		151 ATERALSWDS	FNTGDCFILD	LGQNIFAWCG	GKSNILERNK	ARDLALAIRD
		201 SERQGKAQVE	IITDGEEPAE	MIQVLGPKPA	LKEGNPEEDI	TADQTNAQAA
		251 ALYKVSDATG	QMNLTKVADS	SPFASELLIP	DDCFVLDNGL	CGKIYIWKGR
		301 KANEKERQAA	LQVADGFISR	MRYSPNTQVE	ILRQGRESPI	FKQFFKNWK
Follistatin-related protein	36,020	1 MWKRWLALAL	VTIALVHGEE	EQRSKSKICA	NVFCGAGREC	AVTEKGEPTC
		51 LCIEQCKPHK	RPVCGSNGKT	YLNHCELHRD	ACLTGSKIQV	DYDGHCKEKK
		101 SVSPSASPVV	CYQANRDELR	RRIIQWLEAE	IIPDGWFSKG	SNYSEILDKY
		151 FKSFDNGDSH	LDSSEFLKFV	EQNETAVNIT	AYPNQENNKL	LRGLCVDALI
		201 ELSDENADWK	LSFQEFLKCL	NPSFNPPEKK	CALEDETYAD	GAETEVDCNR
		251 CVCSCGHWVC	TAMTCDGKNQ	KGVQTHTEEE	MTRYAQELQK	HQGTAEKTKK
		301 VNTKEI				
Pigment epithelium derived factor	46,507	1 MQTLVLLLWT	GALLGHGSSQ	NVPDSSQDSP	APDSTGEPVV	EEDDPFFKAP
		51 VNKLAAAVSN	FGYDLYRLRS	GAVSTGNILL	SPLSVATALS	ALSLGAEQRT
		101 ESVIHRALYY	DLINNPDIHS	TYKELLASVT	APEKNFKSAS	RIVFERKLRV
		151 KSSFVAPLEK	SYGTRPRILT	GNPRIDLQEI	NNWVQAQMKG	KIARSTREMP
		201 SALSILLLGV	AYFKGQWATK	FDSRKTTLQD	FHLDEDRTVR	VPMMSDPKAI
		251 LRYGLDSDLN	CKIAQLPLTG	SMSIIFFLPL	TVTQNLTMIE	ESLTSEFVHD
		301 IDRELKTIQA	VLTVPKLKLS	YEGDVTNSLQ	DMKLQSLFES	PDFSKITGKP
		351 VKLTQVEHRA	AFEWNEEGAG	TSSNPDLQPV	RLTFPLDYHL	NRPFIFVLRD
		401 TDTGALLFIG	RILDPSST			
Phosphoglycerate kinase (EC 2.7.2.3)	45,023	1 MSLSNKLTLD	KLDVKGKRVV	MRVDFNVPMK	NNQITNNQRI	KAAVPSIKFC
		51 LDNGANSVVL	MSHLGRPDGV	PMPDKYSLEP	VAAELKSLLG	KDVLFLKDCV
		101 GSEVENACAN	PAAGTVILLE	NLRFHVEEEG	KGKDASGNKV	KAEPAKIDAF
		151 RASLSKLGDV	YVNDAFGTAH	RAHSSMVGVN	LPQKAGGFLM	KKELNYFAKA
		201 LESPERPFLA	ILGGAKVADK	IQLINNMLDK	VNEMIIGGGM	AFTFLKVLNN
		251 MEIGTSLYDE	EGAKIVKDLM	TKAEKNGVKI	TLPVDFVTAD	KFDENAKTGQ
		301 ATVASGIPAG	WMGLDCGTES	SKKYAEAVAR	AKQIVWNGPV	GVFEWEAFAR
		351 GTKSLMDEVV	KATSRGCITI	IGGGDTATCC	AKWNTEDKVS	HVSTGGGASL
		401 ELLEGKVLPG	VDALSNV			
Transcobalamin II precursor	47,958	1 MELLKALLLL	SGVLGALAEF	CVIPKMDGQL	VEKLGQRLLP	WMDRLSSEQL
		51 NPSIYVGLRL	SSMQAGTKEN	LYLHNLKLHY	QQCLLRSTSS	DDNSGCQTKI
		101 SGGSLALYLL	ALRANCELLG	SRKGDRMVSQ	LKWFLEDEKK	AIGHHHEGHP
		151 HTSYYQYGLS	ILALCVHRKR	VHDSVVGKLL	YAVEHDYFTY	QGHLSVDTEA
		201 MAGLAFTCLE	RFNFNSDLRP	RITTAIETVR	EKILKAQAPE	GYFGNIYSTP
		251 LALQMLMTSP	GVGLGPACLK	ARKSLLLSLQ	DGAFQNPMMI	SQLLPVLNHK
		301 TYLNLISPDC	QAPRVMLVPA	TEDPVHLSEV	SVTLKVSSVL	PPYERTVSVF
		351 AGASLEDVLN	RARDLGEFTY	GTQASLSGPY	LTSVLGKEAG	DREYWQLLRV
		401 PDTPLLQGIA	DYKPKNGETI	ELRLVKM		

[a]The peptides detected by liquid chromatography–tandem mass spectrometry analysis are underlined.

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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-retinalautoimmunity 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-retinalautoantibodies 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, polysaccarides, 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, α1-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-retinalautoantibodies in the sera of patients with AMD (17, 18). Anti-retinalautoantibodies 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-retinalautoimmunity in late onset monkeys was subsequently examined. Anti-retinalautoantibodies 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 μm 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 μl of the same buffer. Cysteine was reduced by adding 20 μl of 50 mM DTT and incubating for 1 h at 37°C. Subsequently, 20 μl 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 μg of trypsin at 37°C overnight. The resultant tryptic peptides were dried, resuspended in 40 μl 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; assembled by AMR Inc., Tokyo, Japan). Peptides were separated on a Magic C18 column (200 μm ID×5 cm, particle size 5 μm, pore size 200 Å; Michrom Bioresources) by using aqueous formic acid/acetonitrile solvents, a flow rate of 3 μl/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, Mammheim, 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 electophoresed 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′-ATGTCTACTGTTCACGAAATCCTG-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 Ni^{2+} 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. 1*A*. 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. 1*B*. 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. 1*C* 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. 1*E*). Drusen that had an eosinophilic inclusion could be observed (indicated by an asterisk in Fig. 1*F*). 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. 1*G*). 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. 1*A* 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. 1*H*). 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. 2*A* and *B*), amyloid P component (Fig. 2*C* and *D*), complement component C5 (Fig. 2*E* and *F*), the terminal C5b-9 complement complex (Fig. 2*G* and *H*), and fluid phase inhibitor of complement cascade, vitronectin (Fig. 2*I* 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. 2*K* 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. 3*A*. 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. 3*B*, white materials in a circle). Drusen were isolated with a tiny needle or a micropipette and transferred into ammonium bicarbonate buffer (Fig. 3*C*, 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. 4*A*. 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. 1*C* is shown in Fig. 4*B*. 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. 4*A*). 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. 4*C*). 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. 4*D*, 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. 4*E*). 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. 4*F*).

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. 5*A*). 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. 5*B*).

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. 1*C–E*) and the other by degenerative changes in RPE cells such as hyperpigmentation, hypopigmentation, and vacuolation (Fig. 1*A*, *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. 1*C* 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 diagnostic 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+} homoeostasis 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. 4*F*). 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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Table 1

Antibodies used in immunohistochemical studies and conditions of antigen retrieval treatments

Antigen	Retrieval Treatment	Primary Antibody		
		Host	Dilution	Supplier
Amyloid P Component	Pro K	Rabbit	200	Dako, Carpenteria, CA
Apolipoprotein E	-	Mouse	200	Biogenesis, Poole, UK
C5	Pro K	Rabbit	200	Dako, Carpenteria, CA
C5b-9	Pro K	Mouse	50	Dako, Carpenteria, CA
MCP	Autoclave	Rabbit	50	Santa Cruz, Santa Cruz, CA
Vitronectin	-	Mouse	100	Chemicon, Temecula, CA

Table 2

Macular status of aged monkeys

Grade	Examined Year			Total	Percentage
	2001	2003	2004		
Normal	45	98	45	188	67.6%
Mild	4	11	15	30	10.8%
Moderate	5	16	10	31	11.2%
Severe	6	17	6	29	10.4%
Total	60	142	76	278	100.0%

Two-hundred and seventy-eight aged female monkeys were examined by fundus scope and classified into 4 grades. Normal: macula with no detectable pigmentary abnormalities. Mild: fewer than 5 yellowish-white spots. Moderate: 5 to 20 spots. Severe: more than 20 spots.