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

PURPOSE. We have previously reported an inherited form of macular degeneration in cynomolgus monkey (*Macaca fascicularis*) with drusen developing at 2 years after birth [1]. In this study the molecular composition of drusen in monkeys affected with age-related form (late onset) and inherited form (early onset) of macular degeneration are both characterized. Involvement of anti-retinal autoimmunity in the deposition of drusen and the pathogenesis of the disease was also evaluated.

METHODS. Funduscopic and histological examinations were performed on 278 adult monkeys (mean age = 16.94 y) for late onset macular degeneration. The molecular composition of drusen was analyzed by immunohistochemical method 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 normal control monkeys by western blot techniques. Immunogenic molecules were identified by 2D electrophoresis and LC-MS/MS. Relative antibody titer value against each antigen was determined by ELISA in sera from 42 affected (late onset) and 41 normal monkeys by ELISA.

RESULTS. Yellowish-white spots in the macular region were observed in 90 (32 %) of the late onset monkeys examined. Histological examination demonstrated that drusen or degenerative 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 kDa and 40 kDa were identified as Aannexin II and μ-crystallin respectively. Relative antibody titer value against annexin II in affected monkeys were significantly higher than control animals (P value < 0.01). Significant difference was not observed in antibody titer value against μ-crystallin, however, several affected monkeys showed considerably elevated titer value (360 – 610 %) compared to the mean for unaffected animals.

CONCLUSIONS. 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 complement cascade at the site of drusen formation.

INTRODUCTION

Age-related macular degeneration (AMD) is the most common cause of legal blindness in people over 60 years of age, and is estimated to affect millions of individuals in industrialized countries. Among people aged over 75 years, mild or early forms occur in nearly 30 % and advanced form in about 7 % of the population [2]. 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 1970's [3]. Aged monkeys spontaneously show macular degenerative changes, such as pigment mottling, hyper- or hypopigmentation and drusen in the macula [4, 5]. The late onset form of macular degeneration in these monkeys is consistent with the phenotype observed in the early stage of human AMDAMD. Thus, macaque monkeys have been suggested as an optimum animal model for AMD [6, 7]. In addition, we have previously reported an early onset macular degeneration in a cynomolgus monkey pedigree maintained at Tsukuba Primate Center [1, 8-10]. For these monkeys the symptoms appear early in life around the age of 2 years, and progress slowly throughout life. The disease has been shown to have autosomal dominant inheritance [1, 11]. These two forms of macular degeneration, late onset and early onset, in monkeys could be extremely valuable models of the early stage of human AMDAMD, 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 human AMDAMD models.

Drusen composition and origin have been analyzed extensively in human AMDAMD. Various of lipids, polysaccarides, and glycosaminoglycans have been identified as constituents [12]. Recent immunohistochemical studies have revealed that drusen contains protein

molecules that mediate inflammatory and immune processes [13, 14]. 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, a1-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, as well as choroidal dendritic cells. These findings have led to the suggestion that immune complex-mediated inflammation damages RPE cells, and choroidal dendritic cells are activated and recruited by injured RPE, while RPE cells respond to control dendritic cell activation by secreting proteins that modulate the immune response. Shedding or endocytosis of cell membranes of injured RPE or dendritic cells are postulated to function as cores for these secreted components to accumulate and form extracellular deposits [12].

Furthermore, the co-distribution of IgG and terminal complement complexes in drusen suggest an immune response directed against retinal antigens and immune complex formation [15]. This hypothesis is supported by the presence of putative anti-retinal autoantibodies in the sera of patients with AMD [16, 17]. Anti-retinal autoantibodies have previously been reported in a number of retinal diseases, including retinitis pigmentosa [18], paraneoplastic retinopathies [19], and retinal vasculitis [20]. 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 [21]. 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 requires 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 were investigated by immunohistochemistry and proteome analysis for comparison with drusen in human AMDAMD. Involvement of anti-retinal autoimmunity in late onset monkeys was subsequently examined. Anti-retinal autoantibodies in sera from the 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 development of possible therapeutic reagents for prevention of drusen formation.

MATERIALS AND METHODS

Maintenance of monkeys

The cynomolgus monkey pedigree with inherited form of early onset macular degeneration was reared in Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases in Tokyo, Japan. All monkeys were treated in accordance with the rules for care and management of the Tsukuba Primate Center [10] 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 Infectious Diseases of Japan. The monkeys used for studies of age-related form of late onset macular degeneration were reared in large-scale breeding facilities in Manila, Philippines (Simian Conservation Breeding & 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 & Research Center, 278

female monkeys ranging from 13 to 25 years old were examined. The mean age was 16.94 years old and the median age was 17 years. The clinical examination was performed after tranquilization by intramuscular injection of 10 mg/kg ketamine-HCl (Ketalar-50; Sankyo, Tokyo). Approximately 20 min prior to examination of the ocular fundi, one drop of a mixture of 0.5% tropicamide and 0.5% phenylephirine 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 fundus camera (TRC50; 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 ºC 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 ºC. Subsequently, the sections were blocked with 5 % skim milk in PBS. The specimens were then reacted with primary antibodies diluted in PBS for 2 hrs at room temperature. Conditions for

antigen retrieval and dilution of primary antibodies for each antigen are shown in Table 1. After washing, 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 processing, sections were examined using a confocal laser scanning microscope (Radiance 2100, BioRad, 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 by 100 mM ammonium bicarbonate buffer (pH 8.0). At magnifications between 20 and 30 diameters, drusen could be observed attached to the surface of Bruch's membrane. Drusen was scraped up with a tiny tungsten needle, the needlepoint of which is 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 micro injector pump (Narishige, Tokyo, Japan). Isolated drusen was stored at -80 ºC until further analyses.

Direct proteomic analysis of drusen components

Ten micrograms of isolated drusen suspended in ammonium bicarbonate buffer was dried and re-dissolved in 20 μl of the same buffer. Cysteine was reduced by adding 20 μl of 50 mM DTT for 1 hr at 37 ºC. Subsequently, 20 μl of 100 mM iodoacetamide was added and the alkylation continued 30 min at room temperature in the dark. The preparation was then digested with1 μg of trypsin at 37 ºC for 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). Peptides were separated on a Magic C18 column (200 μm IDx 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 separated from unaffected monkeys (age?4 years 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 16,000g 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; BioRad), 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-Tween 0.1 %. Sera collected from 20 affected and 10 age-matched control monkeys were used in this study. After incubation for 1 hr at room temperature, the strips were washed four times with PBS tween 0.2 %, 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 tween 0.1% 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 separated from unaffected monkeys. Subsequently, the total protein solution was precipitated by changing solvent composition in a step-wise fashion such that a set of 7 protein fractions was produced. These procedures were carried out using 2-D Fractionation kit (Amersham Biosciences, Buckinghamshire, UK). 8 μgEight 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 overnight at 4 ºC overnight. To the dialyzed protein solution was then added 4× sample buffer additives containing 200 mM DTT, 16 % CHAPS, 0.8 % carrier ampholytes. The samples were separated by 2-D electrophoresis. One hundred microgram protein was loaded on immobilized pH gradient (IPG) strips (pH 3-10, 4-7, 7 cm; BioRad) by in-gel rehydration for overnight at 20 ºC 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 hr 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, merged with that of protein spots visualized by SYPRO Ruby (BioRad), 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 hr at 37 ºC, and the extraction step was performed with 5 % formic acid in 50 % acetonitrile. The extracted peptides were pooled and dried. After resuspending 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 frame 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 induced with isopropyl-ß-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 value titer

The purified recombinant protein was diluted (0.5 μg/ml) with sodium bicarbonate buffer (pH9.6), and immobilized in 96-well immuno-plates (Nalge Nunc, Rochester, NY). After washing with 0.05 % Tween 20 in PBS, the sample wells were blocked with sodium bicarbonate buffer containing 3 % BSA for 2 hr 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 hr 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 (BioRad) 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 years) were funduscopically examined three times from 2001 to 2004. The fundus appearance typical of a monkey with the late onset macular degeneration is shown in Fig. 1-(a). Fine yellow-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 fluorescein angiography (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 segments 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 the early onset macular degeneration were examined. Clinical and histological findings for drusen in the inherited form of macular degeneration were