previously described [1]. Serial sections of the affected retinas with drusen were incubated with antibodies directed against proteins known to be present in drusen in AMD [13] (Table 1). All drusen in both age-relatedlate onset and inherited forms ofearly onset macular degeneration were heterogeneously bound by antibodies directed against apolipoprotein E (Fig.2-a, b), amyloid P component (c, d), complement component C5 (e, f), the terminal C5b-9 complement complex (g, h), and fluid phase inhibitor of complement cascade, vitronectin (i, j). The membrane-associated inhibitor of complement activation, membrane cofactor protein, was localized in membranous forms along the boundaries between drusen and RPE (k, 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 4 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 micro pipette, and transferred into ammonium bicarbonate buffer (Fig. 3-c, arrows). The obtained protein yield was between 10 and 20 $\mu$g per preparation. The isolated drusen (10 $\mu$g) were digested with trypsin, and analyzed by LC-MS/MS. As a result, we identified 60 proteins from three separate preparations and LC-MS/MS 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) [22]. 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, 7 proteins represented superfamily in which other family member were known constituents of drusen in human AMDAMD, 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 identified to, or related to, known component 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 aged -matched unaffectedcontrol 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 were 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 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 $\mu$-crystallin, but the 50 and 60 kDa proteins could not be identified.

*Evaluation of autoantibody by ELISA using recombinant antigens*

Relative antibody titer value against annexin II or $\mu$-crystallin in sera collected from 42 affected monkeys with late onset macular degeneration and 41 age-matched unaffectedcontrol animals were determined by ELISA. The purified recombinant annexin II could be observed on SDS-PAGE gel at approximate size of 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 $\mu$-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 value against annexin II in affected monkeys was significantly higher than those in control animals (P value $< 0.01$) (Fig. 4-e). Seven affected monkeys showed more than twice the mean value titer of the control group. On the other hand, relative antibody titer value against $\mu$-crystallin did not show significant difference between affected and unaffected monkeys. However, several affected monkeys showed considerably high titer value (360 – 610 %) (Fig. 4-f).

*Expression of annexin II in monkey retina*

The protein localization of annexin II in the retina was analyzed by western blotting. Annexin II was present in protein extract from whole retina or choroid, but 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

Age related macular degenerationAMD is the leading cause of blindness in individuals over the age of 650 in industrialized countries. Limited access to the human retinal tissues and the lack of good animal models in species with well-developed macula makes this disease difficult to study. Previous attempts to simulate human AMDAMD in experimental animals such as rodents through high-fat diets and phototoxicity [23,24], senescence acceleration [25], or candidate gene manipulation [26-28] haves 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 [3-7]. We have recently reported a monkey pedigree with early onset macular degeneration where drusen are observed less than two years 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 to hypothesize the involvement of anti-retinal autoimmunity in the etiology of AMD by identification of an autoimmunoantigen expressed in RPE cells.

The study was initiated from clinical observation of late onset macular degeneration in Ccynomolgus 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 is characterized by the formation of drusen (Fig. 1-c, d, e), and the other by degenerative changes of RPE cells such as hyperpigmentation, hypopigmentation, and vacuolation (Fig. 1-a, b, h). These vacuolated and lipid-laden RPE cells are observed as pigmentary abnormalities in fundus photographs and hyperfluorescence in FA explaining the presence of drusen like spots in the retina due to the window defect of these cells. True drusen and lipid-laden RPE cells were difficult to distinguish and diagnose as drusen-like spots in both types of late onset monkeys. 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 1-d. This resembles the "non-geographic atrophy" reported in the Chesapeake Bay Waterman Study [29], or the "pigmentary abnormality" of International Classification and Grading System for Age-related Maculopathy and Age-related Macular Degeneration [30]. In none of affected monkeys we have examined, have we observed

choroidal neovascularization, disciform scarring, geographic atrophy, or other advanced pathologic changes characteristic of later stage human AMDAMD. 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) [22], 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 [31,32]. 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 following photo-oxidation [33], and also in sera from AMD patients [34]. 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 monkeys with late onset macular degeneration in monkeys, as has been suggested in human AMDAMD.

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 human AMDAMD (Fig. 2). Drusen of late onset monkeys are observed in animals ranging in age from 13 to 25 years, while drusen in early onset monkeys may appear around the age of 2 years [1]. Recently, Ambati *et al.* reported knockout mice lacking monocyte chemoattractant protein-1 or its cognate gene C-C chemokine receptor which developed cardinal features of human AMDAMD, including accumulation of lipofuscin in RPE, photoreceptor atrophy, and choroidal neovascularization [35]. 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, which leading to abnormal complement activation. A similar genetic defect which 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 casual events that trigger abnormal complement activation and subsequent drusen formation in AMD.

Involvement of anti-retinal autoimmunity against annexin II and $\mu$-crystallin for late onset macular degeneration monkeys was also described in this study (Fig. 4). Immunohistochemical analysis failed to detect these proteins in retinal cross sections in this study, however, a previous report localized annexin II to the basal plasma membrane of the RPE [22]. Moreover, this report identified annexins (annexin I, II, V and VI) as drusen components in human AMDAMD. Annexin V was also identified in this study by proteome analysis of drusen composition as shown in Table 3. A possible pathological pathway where by autoimmunity against annexin II could contribute to drusen formation in 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; 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 [36]. Annexin I is known to function as an anti-inflammatory mediator because of its response to glucocorticoids [37] and its activities in several animal models of inflammation [38, 39]. Furthermore, high levels of anti-annexin autoantibodies have also been reported in sera from patients with the common chronic inflammatory disease, rheumatoid arthritis (RA) [40, 41]. Continuous production of annexin autoantibodies which 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 [36, 42]. 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 [43]. Autoantibodies against annexin II could be one such inhibitory factor and may contribute to the pathogenesis of diseases in late onset monkeys and human AMD.

Several affected monkeys showed considerably high antibody titer values against $\mu$-crystallin compared to 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. $\mu$-Crystallin is a taxon specific crystallin first described as a lens protein in several Australian marsupials [44]. It binds NADPH is related to enzymes involve in aminoacid metabolism, and is also expressed in photoreceptors and RPE [45]. 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 [22]. Our observations (Table 3) confirm crystallins are present in monkey drusen (Table 3). It can be hypothesized that injured RPE cells shed their cell membranes with cytoplasmic $\mu$-crystallin into sub-RPE space exposing them as new autoantigen. However $\mu$-crystallin expression was limited in the retina of normal control monkey compared to RPE (data not shown). One explanation of this discrepancy is that RPE cells compromised by some physical or metabolic stress might newly express $\mu$-crystallin, therefore, in this case, the appearance of anti-$\mu$-crystallin autoantibodies may be considered as the secondary event after RPE cell injury

caused by chronic complement attack.

It still remains unclear whether autoantibodies against annexin II or $\mu$-crystallin are the initial cause of the disease. It is possible that autoimmunity against these, or other proteins might be the most critical event in the retina because annexin II is a ubiquitous protein and $\mu$-crystallin is also expressed in brain, muscle, and kidney [46]. Detailed clinical information on immunity in individual monkeys is essential to determine the primary event 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 suggests the involvement of antigenic molecules anti-retinal autoimmunity in AMD. Defining the AMD-related autoantibodies may provide possible diagnostic tool for the early detection and management of human AMDAMD.

ACKNOWLEDGEMENTS

This work was supported by research grant, Research on Measures for Intractable Diseases, Ministry of Health, Labor and Welfare of Japan (Iwata) and by the fellowship of the Promotion of Science for Japanese Junior Scientists (Umeda). We appreciate Dr. Samuel Zigler Jr. of the National Eye Institute, NIH for the critical reading of the manuscript.

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