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Plasma Proteome Analysis on Cynomolgus Monkey (*Macaca Fascicularis*) Pedigrees with Early Onset Drusen Formation

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Abstract: The central region of the primate retina is called macula. The fovea is located at the center of the macula, where the photoreceptors are concentrated to create neural network adapted for high visual acuity. Damage to the fovea by macular dystrophies and age-related macular degeneration (AMD) can reduce the central visual acuity. The molecular mechanisms leading to these diseases are most likely dependent on the proteins in macula differ from that in peripheral retina in expression level. Previously, we reported an early onset macular degeneration with drusen in cynomolgus monkey pedigrees. These monkeys show similar fundus findings of early stage of AMD at 2 years after birth. To elucidate mechanism of drusen formation and to find disease biomarkers for early stage of AMD, we performed plasma proteome analysis. Plasma samples were collected from four affected and control monkeys within the same pedigree. Successful fractionation of the plasma proteins by ProteoMiner and Gelfree8100 were confirmed by SDS-PAGE. Total of 245 proteins were identified from eight samples. From the results of spectral counting, we selected some proteins, Apolipoprotein E, Histidine-rich glycoprotein, and Retinol-binding protein 4 as candidate proteins that would be related with drusen formation. Candidate proteins would be potentially beneficial as biomarkers for human AMD. One of the identified proteins, Apolipoprotein E (ApoE), is structural component of drusen and also related with other neurodegenerative disease like Alzheimer disease. In this plasma proteome analysis, ApoE would be one of the possible factors of early drusen formation in these cynomolgus monkey pedigrees.

Key words: age-related macular degeneration, drusen, mass spectrometry, plasma, proteome

Introduction

Age-related macular degeneration (AMD) is a leading cause of irreversible visual loss in elderly populations [20]. Drusen is accumulation of debris-like material between the retinal pigment epithelium (RPE) and Bruch's membrane. Although the biological basis of the process is still unknown, development of soft drusen is one of main findings of AMD. Previously, we reported an early-onset of drusen formation in a cynomolgus monkey pedigree [14, 17, 18] (Fig. 1). These monkeys show similar fundus findings of early stage of age-related macular degeneration at two years after birth [19]. The symptoms appear early in life around the age of two years old and progress slowly throughout life and focal ERG were significantly affected in monkey with heavy drusen. The disease has been shown to have autosomal dominant inheritance. These forms shown in monkeys

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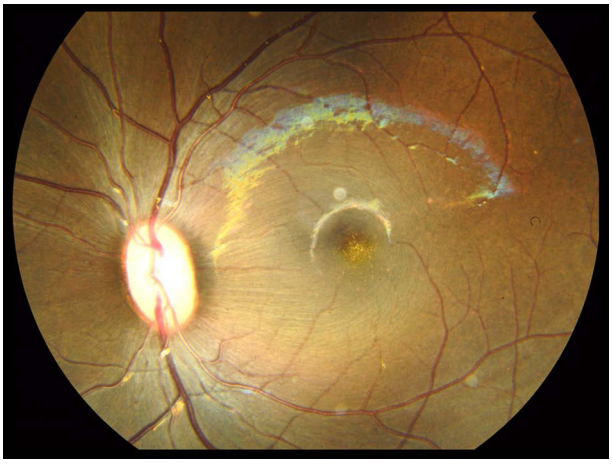

Fig. 1. Fundus photograph of affected monkey.
The number of drusen increase significantly during the age of 2 to 5 years old. The number of drusen is generally bilaterally equal.

could be extremely valuable models of the early stage of AMD, especially for elucidating the mechanism of drusen formation. In this study, we performed plasma proteome analysis to elucidate mechanism of drusen formation and to find disease biomarkers for early stage of AMD. Abundance of plasma proteins spans about 12 orders of magnitude at log scale and 22 major proteins account for approximately 99% of plasma proteins [3]. Plasma proteome analysis often encounters difficulty of detecting low molecular weight proteins due to the characteristics of plasma. To solve this problem, we used ProteoMiner Enrichment Kit and GELFREE8100 fractionation system in this study.

Materials and Methods

Animals and plasma samples

All experiments on cynomolgus monkeys were approved by the Animal Ethics Committee of the Tsukuba Primate Research Center (TPRC) and were conducted in accordance with The Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The monkey blood samples were harvested in citric acid collection tube. After centrifugation at 2,000 rcf for 10 min, plasma was separated from blood cells. Plasma was stored at −80°C until use.

Two Methods of Sample Preparation

To decrease dynamic range of plasma protein concentration, or to detect low molecular weight proteins undisturbedly, crude plasma samples were first treated with two different methods, peptide ligand library or molecular weight partitioning with liquid phase recovery (Fig. 2).

Peptide ligand library

ProteoMiner is based on treatment of complex protein samples with a large, highly diverse library of hexapeptides bound to chromatographic supports. Each unique hexapeptide binds to a unique protein sequence. Because the bead capacity limits binding capacity, high-abundance proteins quickly saturate their ligand and excess proteins are washed out during the procedure. On the other hand, low-abundance proteins are concentrated on their specific ligands, thereby decreasing the dynamic range of proteins in the samples [4, 8].

Forty micrograms of treated samples were separated on 12% acryl amide SDS-PAGE gel and visualized by Coomassie Brilliant Blue (CBB). Each lane was cut into 35 homogenous slices and their width was about 1 mm. Each gel piece was cut into approximately one cubic millimeter and washed with 50 mM ammonium bicarbonate/50% acetonitrile until CBB was destained. The gel pieces were rinsed with distilled water, and incubated with acetonitrile for 20 min. Then supernatant was discarded and the gel pieces were completely dried before incubation with 10 mM DTT in 100 mM ammonium bicarbonate for 45 min at 56°C. The supernatant was discarded and the pieces were incubated in the dark with 55 mM iodoacetamide in 100 mM ammonium bicarbonate for 30 min at room temperature. The supernatant was discarded, and the gel pieces were washed with 50% acetonitrile, 100% acetonitrile, and 100 mM ammonium bicarbonate. After drying the gel pieces completely, we proceeded trypsin digestion by sequencing grade modified trypsin solution (12.5 ng/μl; Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate. The digestion was performed at 37°C for 12 h. The extraction step was performed once with 25 mM ammonium bicarbonate, twice with 5% formic acid, and finally with distilled water. The extracted peptides were collected in one microtube and dried up by centrifugal drying and stored at −20°C until just before use.

Molecular weight partitioning with liquid phase recovery

The GELFREE8100 (Protein Discovery, Inc., USA) is a protein fractionation system designed to maximize

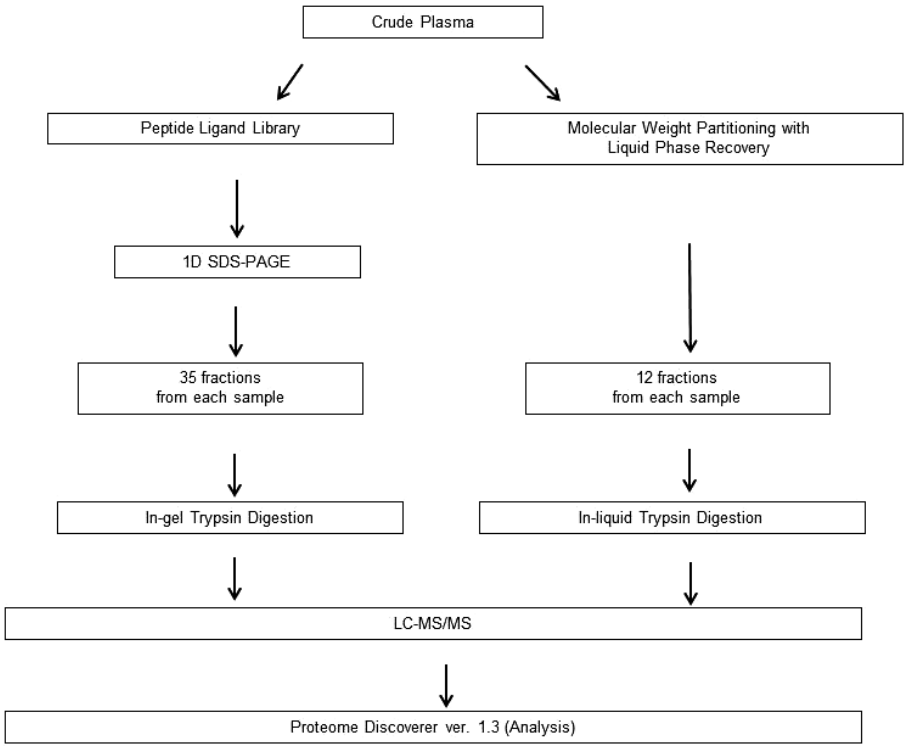

Fig. 2. Experimental scheme of the plasma proteome analysis.

protein recovery during molecular weight based fractionation. The system is comprised of single-use, 8-sample capacity cartridges and a bench top GELFREE-Fractionation Instrument. During separation, a constant voltage is applied between the anode and cathode reservoirs, and each protein mixture is electrophoretically driven from a loading chamber into a specially designed column gel. Proteins are concentrated into a tight band in a stacking gel, and separated based on their respective electrophoretic mobility in a resolving gel. As proteins elute from the column, they are trapped and concentrated in liquid phase in the collection chamber, free of the gel. The instrument is then paused at specific time intervals, and fractions are collected using a pipette. This process is repeated until all desired fractions have been collected [16, 22]. One hundred fifty micrograms of plasma samples were first desalted by Zeba Spin Desalting columns. Desalted samples were loaded on each loading chamber of 12% Tris-acetate cartridge (mass range, 3.5 kDa-50 kDa) and concentrated in stacking gel. Finally, proteins are fractionated in resolving gel and collected in collection chamber as 12 liquid phase samples per one crude plasma. Thermo Detergent Removal Spin Columns cleaned up SDS from each collected sample. These samples were incubated with DTT solution for 60 min at 37°C and then incubated in the dark with 1/20 (w/w) iodoacetamide for 30 min at 37°C. Finally, 200 ng/μl trypsin in 50 mM acetic acid was added to samples and tryptic digestion was performed at 37°C overnight. All solution were dried up and resuspended with 20μl of aqueous 0.1% trifluoroacetic acid/2% acetonitrile.

LC-MS/MS analysis

LC-MS/MS was performed with a combined Paradigm MS4 (Michrom BioResources, Auburn, CA, USA) and an ESI mass spectrometer (LCQ Deca XP plus, Thermo Fisher Scientific, Yokohama, Japan; assembled by AMR Inc. Tokyo, Japan). For the LCQ analysis, sample peptides were separated in nano column (AMR Inc.) with solvent A (2% acetonitrile/0.1% formic acid) and B (90% acetonitrile/0.1% formic acid) at flow rate 2.0 μl/min. The identification of the proteins from the MS/MS spectra was performed using Proteome Discoverer ver. 1.3 (Thermo Fisher Scientific) and UniProtKB/Swiss-Prot database released on December 14, 2011, which was preliminarily extracted by the species "human". We also generated reversed database (decoy database) based on

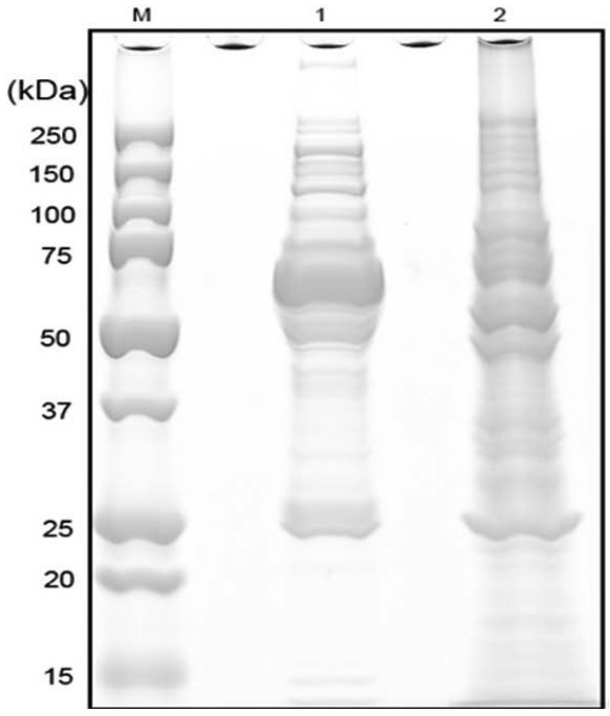

Fig. 3. Comparison of SDS-PAGE comparison of crude plasma versus ProteoMiner fractionated proteins.
Twenty micrograms of proteins were loaded on 12% acrylamide gel and stained by Coomassie Brilliant Blue. Lane M: Precision Plus Protein Standards; Lane 1: Crude plasma, Lane 2: Eluate from ProteoMiner.

correct database. List generation and database searches were performed with the following parameters: maximum missed cleavage sites, 2; precursor mass tolerance, 2.0 Da; fragment mass tolerance, 0.8 Da; static modification, carbamide-methylation (+57.02 Da) for cysteine; dynamic modification, oxidation (+16.00 Da) for methionine. The peptide sequences were filtered by false discovery rate (FDR) and the threshold level of FDR was<5%. Finally, semi-quantitative analysis was performed by an approach referred to as spectral count. Spectral count was suggested that it showed excellent correlation with protein amount. On the other hand, peptide count correlates poorly with protein amount and sequence coverage does not correlated with protein amount [11].

Results

Peptide ligand library treated with ProteoMiner decreasing dynamic range of protein concentration in plasma samples and Gelfree8100 also indicated successful fractionation of low molecular weight proteins (Figs. 3 and 4). In this study, we identified total 245 proteins from 8 plasma samples, 186 proteins in ProteoMiner and 106 proteins in GelFree8100 and also identified 195 proteins were identified from disease samples and 156 proteins from normal samples. We also performed semi-quantitative analysis with spectral count. Each protein was quantified with consideration of its amino acids length and amount of files of peptide spectrum in this method. High absolute value of abundance ratio of each protein (R_{sc}) means that there is difference in amount of protein between disease and control samples [23]. We selected some proteins, Apolipoprotein E, Histidine-rich glycoprotein, and Retinol-binding protein 4 as candidate proteins. Apolipoprotein E is related with drusen, Histidine-rich glycoprotein is related with regulation of angiogenesis and with VEGF causing choroidal neovasculization [15], and Retinol-binding protein 4 is related with disease characterized by retinal degeneration.

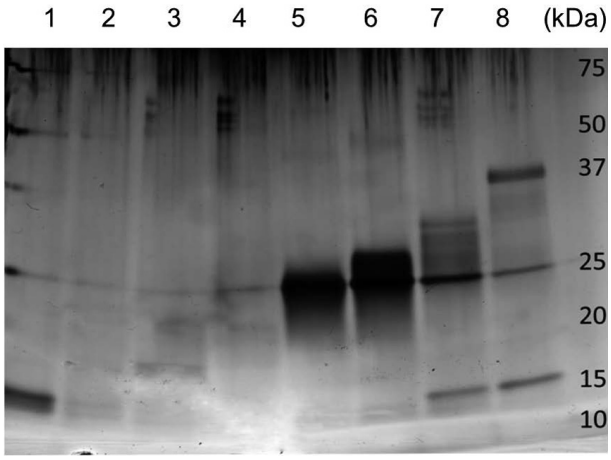

Fig. 4. Silver staining of Gelfree8100 fractionation.
Numbers mean order of collection. Desalted samples were loaded on each loading chamber of 12% Tris-acetate cartridge (Mass range, 3.5-50 kDa). Lane 1: 1st fractionated sample; Lane 2: 2nd fractionated sample; Lane 3: 3rd fractionated sample; Lane 4: 4th fractionated sample ; Lane 5: 9th fractionated sample; Lane 6: 10th fractionate sample; Lane 7: 11th fractionated sample; Lane 8: 12th fractionated sample.

Discussion

In this study, plasma proteins of cynomolgus monkeys with early-onset drusen formation were identified by two different sample preparation methods. More proteins were detected from samples treated by ProteoMiner. This

Table 1. Candidate Proteins identified in samples dealed with ProteoMiner

Protein name	Database accession No.[a)]	MW[b)] (kDa)	No. of spectrum		R_{SC}
			Disease	Normal	
Complement factor H-related protein 5	Q9BXR6	64.4	0	6	3.187
Apolipoprotein B-100	P04114	515.3	3	0	–1.120
Complement C3	P01024	187.0	4	0	–1.425
Histidine-rich glycoprotein	P04196	59.5	14	1	–2.119

a) Accession No. corresponds to UniProtKB/Swiss-prot database. b) MW are theoretical score.

Table 2. Candidate Proteins identified in samples dealed with Gelfree8100

Protein name	Database accession No.[a)]	MW[b)] (kDa)	No. of spectrum		R_{SC}
			Disease	Normal	
Retinol-binding protein 4	P02753	23.0	7	23	1.471
Apolipoprotein E	P02649	36.1	7	2	–1.479
Histidine-rich glycoprotein	P04196	59.5	9	1	–2.330

a) Accession No. corresponds to UniProtKB/Swiss-prot database. b) MW are theoretical score.

would be due to the difference of sample state. In the liquid phase samples, some reagents were added in the process of trypsin digestion. These impurities interrupted LC-MS/MS analysis.

We selected some proteins as candidates that had high absolute value of R_{sc} and also thought to be related with retina or AMD (Tables 1 and 2). One of the candidates' proteins is Apolipoprotein E (ApoE), a cholesterol transporter. ApoE travels on small, high-density lipoproteins that export cellular cholesterol from brain and macrophages as well as larger apolipoprotein B-containing lipoproteins that circulate in plasma [12]. Johnson *et al.* described a retinal pigment epithelium (RPE) culture system exhibiting secretion of drusen component ApoE and activation of systemically derived complement, a pathway fingered in AMD by multiple genetic association studies [10]. Curcio *et al.* said that RPE secretes ApoB-lipoprotein particles of unusual composition into Bruch's membrane, where they accumulate with age eventually forming a lipid wall, a precursor of basal linear deposit, the lipid-rich lesion associated with AMD [6]. ApoE is also related to age-related neuronal degenerative disease, like Alzheimer disease (AD). Although drusen is not necessarily seen in fundus of AD patients, a starting of neuronal degeneration caused by aggregation of amyloid-β would be similar to that of RPE degeneration caused by deposition of drusen. One of major risk for AD is ε4 allele of the ApoE gene. APOE4 contributes to pathogenesis of AD by modulating the metabolism and aggregation of amyloid-β peptide which is content of drusen [9].

Confirmed drusen proteins include complement terminal complex C5b-9, complement factor H (CFH), vitronectin, TIMP-3 and apolipoproteins (E, B, and A-1) [5]. Genes for many drusen molecules are expressed by RPE, retina or both [13]. The synergy between complement localization in drusen and association of CFH sequence variants and other genes with AMD pointed strongly to a role for complement in the disease [2]. A large cholesterol-rich lipoprotein secreted by the retinal pigment epithelium (RPE) and accumulating throughout adulthood within Bruch's membrane is hypothesized to be a major trigger for age-dependent complement activation [6, 21].

A relationship between AMD and the concentration of plasma apolipoproteins has been sought repeatedly since the early 1960s [7], but a strong positive correlation between disease status and plasma lipoprotein levels remain to be well explained. Because ApoE is the structural component of drusen [1, 5], and basal linear deposits and drusen would become trigger for age-dependent complement activation, this plasma proteome analysis indicates that ApoE would be one of the possible factor of early drusen formation in these cynomolgus monkey pedigrees.

Acknowledgments

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