blotting was performed to observe the distribution of spots. Figure 6 showed an additional spot in the acidic position (arrow) on the macula membrane compared with peripheral retina. γ-synuclein, a protein known to be up-regulated in cancer cells (33), showed 2.9-fold higher expression in the macula. E-FABP, a reactive lipid scavenger (34), showed 1.9-fold higher expression in the macula. Tropomyosin1a chain Br-1 and Br-3 showed 1.8-fold higher expression in the macula than in the peripheral retina, while tropomyosin1a chain TM311 showed 8-fold higher expression in the macular (Fig. 5-5 & 5-6). Tropomyosin1a chain has been reported in many isoforms by alternative splicing (35). MS/MS data from spot M4 (Fig.1) identified peptide sequence CAELEEELK (Table 2), which corresponded to isoform 1 (skeletal muscle type) or isoform 5 (brain type, TMBr-3) of tropomyosin1a chain in the UniProtKB/Swiss-Prot database.

Based on these data, western blotting was performed using two antibodies for tropomyosin1a chain isoform 1 and 5. The anti-tropomyosin antibody TM311 detects 19 amino acids in exon 1a of tropomyosin gene family in mammalian tissues, viz., alpha-, beta-, gamma-, delta- tropomyosin, including the skeletal muscle type but it does not detect brain type (TMBr-3). The other antibodies used were specific to the brain isoforms TMBr-1 and TMBr-3. hnRNPs A2/B1 had 1.5-fold higher expression in the macula, while hnRNPs C1/C2, had 2.6-fold higher expression in the macula than peripheral retina (Fig.

5-7 & 5-8). hnRNPs A2/B1 is known to be up-regulated in carcinoma cells (36) and hnRNPs C1/C2 for amino acid modification by oxidative stress (37). hnRNPs A2/B1 and hnRNPs C1/C2 products are known as alternative splicing variants. A unique amino acid sequence of hnRNP B1 isoform was identified from spot M37 and M38 but other hnRNPs A2/B1 and all hnRNPs C1/C2 spots contained only common amino acid sequences of each variant.

Tissue localization of macula enriched proteins

To determine the location of the 7 proteins in the macula, immunohistochemistry was performed using antibody against each protein (Fig. 7). Arrestin-C was detected in photoreceptor outer segments and outer plexiform layer (Fig. 7B) as reported (31). γ-synuclein was located in the retinal ganglion cells and the nerve fiber layer. E-FABP was detected throughout the sensory retina except the photoreceptors and RPE cells (Fig. 7E). Tropomyosin Br-1 and Br-3 were located in the photoreceptor inner segments and outer plexiform layer (Fig. 7F), while TM311 was detected in the choroidal layer (Fig. 7G). hnRNPs A2/B1 and hnRNPs C1/C2 were located in the nucleus of a number of retinal cells including the cells in the retinal ganglion cell layer, the inner nuclear layer, the outer nuclear layer, and the RPE layer. However, a difference in the signal intensity was

observed between hnRNPs A2/B1 and hnRNPs C1/C2.

Discussion

Comparison of the different protein levels in the macula and peripheral retina was determined by proteomic analyses, and the results showed a number of quantitatively abundant proteins in the primate macula. One of the proteins was arrestin-C known to be specifically expressed in cone photoreceptors, a cell type highly concentrated in the macula (4). The identification of arrestin-C indicated that this proteomic approach was methodologically effective for the identification of macula enriched proteins. Another example is 3'(2'),5'-bisphosphate nucleotidase 1 previously identified by SAGE study was reported to be highly expressed in cone photoreceptors (7), and our results identified it as another macular enriched protein. Guanylate kinase (Table 2, M3), which catalyzes the phosphorylation of GMP(38) and is essential for recycling cGMP in the photoreceptors (39), was also identified as one of the macular enriched protein.

γ-synuclein, a member of the synuclein family, was identified as a macular specific spot in the 2D-gel. Previous SAGE study also showed that γ-synuclein was highly expressed in the macula (6). Immunostaining showed that this protein was predominantly

located in the retinal ganglion cells and nerve fiber layer (Fig. 7D), which is also consistent with a previous study (40). These findings are in agreement with the high concentration of retinal ganglion cells in the macular area. γ-synuclein was initially identified as a breast cancer specific gene 1 by high throughput differential cDNA sequencing (33). This protein was expressed in peripheral nervous system, brain (41), and in ocular tissues (40). γ-Synuclein knockout mice are viable, fertile, with no abnormal behavior compared to wild type mice (42) with morphologically normal retina (43). The cellular function of γ-synuclein is still unknown, but an over-expression of synoretin, a bovine ortholog of γ-synuclein, activates the Elk-1 signal transduction pathway (44) and the chaperonic activity (43). Snyder et al. hypothesized that γ-synuclein is the regulator of 20S proteosome degradation and has a role in cellular response to oxidative stress because 20S ubiquitin-independent proteosomal pathway is responsible for the degradation of 70-80% of all mildly oxidized proteins (45). In the macula, γ-synuclein is likely to play an important role in the degradation of oxidized proteins.

Six macula specific mitochondria proteins were identified in this study. Mitochondria have been recognized as one of the major cellular generators of reactive oxygen species through electron transport chain reactions (55). Oxidative stress is believed to be important in the aging process and pathogenesis of diseases including AMD (56).

Photoreceptors densely localized in the macula have higher concentration of mitochondria with 2 to 3-fold greater oxygen consumption compared to the inner retina (46) and the distribution and morphological differences of mitochondria in the different regions of the retina have been reported (47). The macula may due to the higher concentration of cones exposed to more oxidative stress than peripheral retina.

Retinal abnormality of the Cu, Zn-SOD knockout mice (57) and Mn-SOD knockdown mice (58) mimic to patients with AMD. A higher demand of ATP production in cones than in rods has been suggested by the analysis of mitochondrial crista structure (59). An accumulation of 4977 bp deleted mitochondrial DNA ($mtDNA^{4977}$), which is mainly caused by free radicals generated from mitochondria in the inner segments, has been shown to increase with age, and at higher concentration in the macula than in the peripheral retina (60). In our results, additional horizontal spots for Mn-SOD were observed in the 2D-western blot (Fig. 6, arrow), indicating a posttranslational modification specific to the macula. The phosphorylation of Mn-SOD by calcium ion has been demonstrated to inactivate enzyme activity (61). The activity of Mn-SOD is essential for the survival (62) and protection of the retina from oxidative stress (63). The presence of numerous phosphorylated forms of Mn-SOD in different region of the retina may explain the preferentially impairment of the macula under high oxidative stress levels.

Addition to these observation, the identified voltage-dependent anion-selective channel protein 1 (VDAC1) is located in the outer membrane of mitochondria (48, 49) and involved in cell volume regulation and apoptosis (X). Age-related maculopathy susceptibility 2 (*ARMS2/LOC387715*), a functionally unknown susceptibility gene for AMD (50-52), encodes a protein which is also localized to the outer membrane of the mitochondria (53, 54). Further functional study of VDAC1 and ARMS2 in correlation with AMD is notable.

E-FABP also plays a role as an antioxidant. 4-hydroxynonenal (4-HNE) is one of the aldehydes produced from lipid peroxidation. E-FABP is a molecular target for 4-HNE modification and functions as an antioxidant protein by scavenging reactive lipids though covalent modification of Cys-120 (34). 4-HNE modified E-FABP has been detected in the rat retina (64) and even higher expression levels in the primate macula to cope with the higher oxidative stress than the peripheral retina. In a transcriptional comparison study between macular and peripheral RPE, the expression of E-FABP was higher in the peripheral than macular RPE (9). Our immunostaining showed that E-FABP was predominantly located in the neural retina except the photoreceptors (Fig. 7E) which is consistent with the results of Kingma et al. (65). Our observation of a higher E-FABP expression in the macula is probably from the neural retina and not the RPE.

Four macula enriched hnRNPs were identified. hnRNPs are RNA-binding proteins predominantly located in the nucleus and function in splicing, transporting, and stabilizing mRNAs (66, 67). hnRNPs A2/B1 and hnRNPs C1/C2 were confirmed to be expressed at significantly higher levels in the macula by western blotting (Fig. 5G and 5H). These proteins were identified from multiple horizontal spots in the same gel indicating the possibility of phosphorylation. hnRNPs C1/C2 has been observed in several posttranslational phosphorylated forms and these forms are dependent on the duration of the oxidative stress (37). It also modulates the affinity of the mRNA binding to hnRNPs C1/C2 (68). The regional specificity of phosphorylation in this protein may affect the pathological process of macula-related diseases. For example, it has been reported that this protein stabilizes the mRNA of amyloid precursor protein (APP) and enhances translation of APP (69). APP and Amyloid beta (Aß) are found in drusen from AMD patients (X). Enrichment and posttranslational modification of hnRNPs C1/C2 may associate with Aß accumulation. In addition to the hnRNPs C1/C2 phosphorylation, hnRNPs A2/B1 phosphorylation is involved in modifying its molecular function. In the central nerve system, phosphorylation of hnRNPs A2/B1 has been considered to be essential for myelination at the axon-glia contact site by mRNA transportation (70).

Tropomyosin1 a chain belongs to a multi-gene family of actin-binding proteins and 5

isoforms are listed in UniProtKB/Swiss-Prot database. In mammals, tropomyosin can give rise to many isoforms by splicing variants and specific isoforms are present in each tissue (35). Although the tropomyosin1a chain and other tropomyosins have been identified in photoreceptors (72), this is the first report to observe distribution of tropomyosin isoforms in macula and to identify brain isoform from photoreceptors. Anti-tropomyosin1a chain antibody TM311 was used for western blotting and immunohistochemistry. TM311 is a monoclonal antibody which detects broad range of alpha-, beta-, gamma- and delta- tropomyosin expressed in both muscle and non-muscle tissue except TMBr-3. By immunostaining, tropomyosin isoforms detected by TM311 were located around the choroidal vessel (Fig. 7G). MacDonald et al. demonstrated that endostatin, a cleavage fragment of collagen XVIII, functions as an anti-angiogenic factor (73) but it requires binding to tropomyosin to gain activity (74). Higher expression of tropomyosin1a chain in the macula has the potential to act as an anti-angiogenic factor for AMD by binding to endostatin.

Phosphorylation of the tropomyosin1a chain has also been described. Using human umbilical vascular endothelial cells, Houle et al. reported tropomyosin phosphorylation by oxidative stress through activation of the ERK pathway. Inhibition of the ERK pathway in the presence of oxidative stress resulted in membrane blebbing which then led to

damage of the vascular endothelium (71).

Spot number M4 corresponded to TMBr-3. TMBr-3 is expressed in all regions of the brain (75, 76) and was detected in the outer plexiform layer and photoreceptor inner segments (Fig. 7F) by double immunostaining with arrestin-C (Fig. 7B). The function of TMBr-3 in the photoreceptor inner segments is still unknown. Tropomyosin has a role in stabilizing actin filaments and regulates actin filaments in competition with other actin binding protein in non-muscle cells (77). Actin filaments located in the photoreceptor connecting cilia are considered the cytoskeletal core necessary for initiating new disk formation (78). Disk turnover is essential for maintenance of normal vision and consequently accumulation of lipofuscin in RPE cells by phagocytosis of disks has been linked to AMD (79). The function of TMBr-3 might involve disk formation by actin binding and rate of lipofuscin accumulation. Furthermore one of peptide fragment, which contains nitro tyrosine, in tropomyosin 1 alpha chain was identified from skeletal muscle (X). Nitration has been considered the potential for pathogenesis of many diseases (X). Brain type isoform also contains same amino acid sequence. In the retina, nitration of tropomyosin might be occurred and be associated with pathogenesis of AMD. Further studies are needed to understand the function of TMBr-3 in the photoreceptors.

Conclusions

Proteomic analyses using 2D gel electrophoresis and LC-MS/MS were performed for comparison of regional differences in primate retina. Twenty-six proteins were identified as macula unique 2D-gel spots and 6 of these proteins were confirmed to be macula enriched by western blotting. In this study, brain type isoform of tropomyosin 1 alpha chain was first reported from retina. Furthermore, additional horizontal spots for Mn-SOD were specifically observed in the macula. Although some of these proteins are derived because of difference in major cell types between the two regions, identification of protein from macular enriched cells is important to understand the pathogenesis of AMD.

Acknowledgement

This study was funded in part by a grant to TI from the Ministry of Health, Labour, and Welfare of Japan and by a grant to HO by the Ministry of Education, Sports, Science and Technology of Japan (KAKENHI 19791305).

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