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Lipid rafts and their possible involvements in neuroimmunological disorders: new research arena

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1. ABSTRACT

Multiple sclerosis (MS) and neuromyelitis optica (NMO) are presumed to be an autoimmune disease in the central nervous system (CNS). Although lipids are most abundant components in the nervous system, it has been believed that cellular and/or humoral immunity to various myelin proteins causes these neuroinflammatory diseases. Recent research advances enable us to study lipids in the membranes and some key molecules involved in various neurological disorders including Guillain–Barré syndrome, Alzheimer's disease, Parkinson's disease, and prion disease, are localized in lipid rafts. In MS and NMO, the key molecules for the pathogenesis or the target molecules for the treatments of MS and NMO are also localized in lipid rafts. Here in this article, we highlight on the possible involvement of lipid rafts in the pathogenesis and treatment of MS and NMO and introduce our recent observation of aquaporin 4 regarding NMO.

2. INTRODUCTION

The plasma membrane in eukaryotic cells contains microdomains that are enriched in certain neutral and acidic glycosphingolipids, sphingomyelin, and sterol (such as cholesterol) to form membrane lipid rafts. These regions exist as planar lipid rafts or caveolae. Planar lipid rafts are continuous with the plane of plasma membrane and caveolae, on the other hand, are morphologically observable flask-like invaginations. Lipid rafts are platforms for many molecular entities, including signaling receptors and ion channels that communicate with extracellular stimuli to the intracellular milieu. Key molecules involved in various neurological disorders including Guillain–Barré syndrome (GBS)(1), Alzheimer's disease (2), Parkinson's disease (3), and prion disease (4), are localized in lipid rafts.

Acidic glycosphingolipid, GM1, is also localized in lipid rafts. Anti-GM1 antibody is commonly associated with a pure motor variant of GBS, characterized by no sensory loss, sparing of the cranial nerves, and predominant distal weakness on extremities. We have previously disclosed that GM1 enhances the action of nerve growth factor (NGF) by enhancing NGF-induced autophosphorylation of high affinity NGF receptor, Trk (5). This enhancing effect of GM1 is at least in part due to the tight association of GM1 with the Trk protein. Our subsequent study revealed that GM1 is essential for the Trk initiated intracellular signal transduction pathway because PC12 cells are unresponsive to NGF biochemically and morphologically following the chemical depletion

of GM1 with ceramide analogue, D-threo-1-phenyl-2-decanoylamin-3-morpholino-propanol, which inhibits glucosylceramide synthase in the cells (6). Thus, GM1 is now well recognized as an essential partner of Trk receptor function. Recently we examined the biological effects of anti-GM1 autoantibodies found in patients with the axonal form of GBS on the Trk-initiated intracellular signaling pathway in a neuronal cell culture system and elucidated the molecular basis of such effects. We found that all sera examined in this study inhibited NGF-induced Trk autophosphorylation responses. Interestingly, these sera induced rapid rearrangement of the Trk receptors from membrane lipid rafts to outside of these structures, although no obvious effects were observed on the distribution of the rafts-marker protein, Ras. These data suggest that anti-GM1 antibodies directly affect the integrity of membrane lipid rafts and exert profound effects on the neuronal survival system (1).

There are few papers studying neuroimmunological diseases of the central nervous system (CNS), i.e., multiple sclerosis (MS) and neuromyelitis optica (NMO) in relation to membrane lipid rafts. Here in this article, therefore, we focus and highlight on the possible involvement of lipid rafts in MS and NMO because some of the key molecules for the pathogenesis or the target molecules for the treatments of MS and NMO are localized in lipid rafts.

3. MULTIPLE SCLEROSIS

Mulltiple sclerosis is presumed to be an autoimmune disease targeting the myelin sheath in the central nervous system (CNS). Cellular and humoral immunity to myelin proteins including myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyteglycoprotein (MOG) causes neuroinflammation (7, 8). Recent studies revealed that non-myelin antigens including neurofilaments, neurofascin, RNA binding proteins, and potassium channels may also contribute to the pathogenesis (9). Although lipids comprise over 70% of myelin sheath, autoimmune responses to lipids have been studied much (far) less than responses to proteins described above because of lack of enabling technologies.

3.1. Exploration of key molecules regarding the pathogenesis of MS

Kanter *et al*. developed large-scale lipid microarrays for detection of autoantibodies present in the serum and cerebrospinal fluid (CSF) (10). By using this method, they identified lipid-specific antibodies against sulfatide, sphingomyelin and oxidized lipids in the CSF derived from MS patients. Sulfatide-specific antibodies were also detected in SJL/J mice with acute autoimmune encephalomyelitis (EAE). Immunization of mice with sulfatide plus myelin peptides resulted in a more severe disease course of EAE, and administration of sulfatide-specific antibody exacerbated EAE. Thus sulfatide and other lipids may contribute to the pathogenesis of autoimmune demyelination.

3.2. αB-crystallin and lipid rafts

van Noort *et al*. examined proliferative responses of human peripheral blood T cells to the complete collection of myelin proteins fractionated by reversed-phase high-performance liquid chromatography (11). Myelin isolated from MS-affected brain contained a single protein fraction to which T cells from MS patients and from healthy controls showed dominant responses. This highly immunogenic protein was identified as αB-crystallin, a small heat-shock protein. They also revealed the presence of oligodendrocytes and astrocytes with raised αB-crystallin expression in active MS lesions, which were not found in unaffected myelin.

αB-crystallin is an intracellular Golgi membrane-associated small heat shock protein. Besides MS, elevated levels of αB-crystallin have been linked in Alexander, Alzheimer, and Parkinson diseases, and age-related macular degeneration. The membrane association of αB-crystallin has been known for more than 3 decades, yet its physiological import has remained unexplained. It has shown that αB-crystallin is secreted from human adult retinal pigment epithelial cells via microvesicles (exosomes), independent of the endoplasmic reticulum-Golgi protein export pathway (12). The presence of αB-crystallin in these lipoprotein structures was confirmed by its susceptibility to digestion by proteinase K only when exosomes were exposed to Triton X-100. Transmission electron microscopy was used to localize αB-crystallin in immunogold-labeled intact and permeabilized microvesicles. The saucer-shaped exosomes, with a median diameter of 100-200 nm, were characterized by the presence of flotillin-1, α-enolase, and Hsp70, the same proteins that associate with detergent-resistant membrane microdomains (DRMs), which are known to be involved in their biogenesis. Notably, using polarized adult retinal pigment epithelial cells, the secretion of αB-crystallin was predominantly from the apical

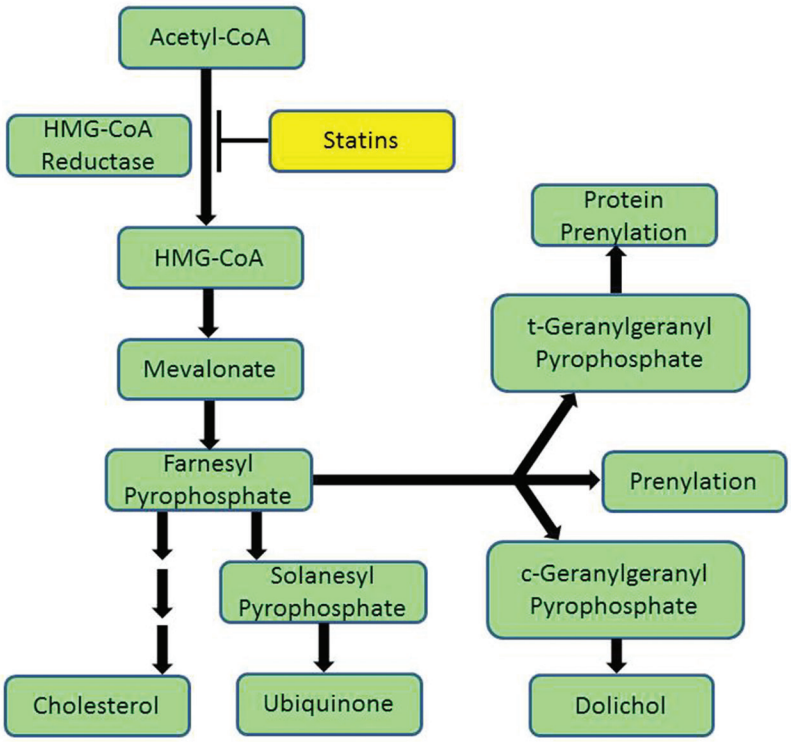

Figure 1. Cholesterol biosynthesis pathway highlighting the biologically active metabolites. HMG-CoA: 3-hydroxy-3-methyl glutaryl coenzyme A. Statins bind and inhibit HMG-CoA reductase.

side. Using OptiPrep gradients, it was demonstrated that αB-crystallin resides in the DRM fraction. The secretion of αB-crystallin is inhibited by the cholesterol-depleting drug, methyl-β-cyclodextrin, suggesting that the physiological function of this protein and the regulation of its export through exosomes may reside in its association with DRMs/lipid rafts.

3.3. Statins

Statins, or 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, mediate their biological effect by inhibiting HMG-CoA reductase, which is an upstream rate-limiting enzyme in the cholesterol synthesis pathway (Figure 1). The consequent reduction in circulating low-density lipoprotein (LDL) cholesterol provided the original rationale for treating cardiovascular disease (13). Cholesterol is a major component of lipid rafts to form platforms where functionally related proteins interact to provide effective signal transduction, such as T-cell receptor and co-stimulatory molecules that form an immunological synapse, and ceramide/sphingomyelin and receptors that mediate cellular signaling (14). Therefore, cholesterol depletion in lipid raft microdomains could alter their structure and function, with a significant effect on cellular activation and signaling pathways (15).

Besides cholesterol-lowering effect, the inhibition of HMG-CoA reductase by naturally existing statins (lovastatin, mevastatin, and simvastatin) and synthetic statins (fluvastatin, atorvastatin and rosuvastatin) inhibits the mevalonate pathway leading to the reduction of its biologically active metabolites, including isoprenoids, dolichol, ubiquinone (16) (Figure 1). Isoprenoids are required for isoprenylation of proteins and for their optimal function (16). Isoprenoids, faresylpyrophosphate and geranylgeranylpyrophosphate bind to proteins during their posttranslational modification and serve an important role in the functional targeting of proteins to different cellular sites (17). Inhibition of isoprenylation is considered to play an important role in the statin-mediated cholesterol-independent pleiotropic effects targeting inflammatory diseases.

Based on these mechanisms of action, multiple clinical trials have been undertaken.

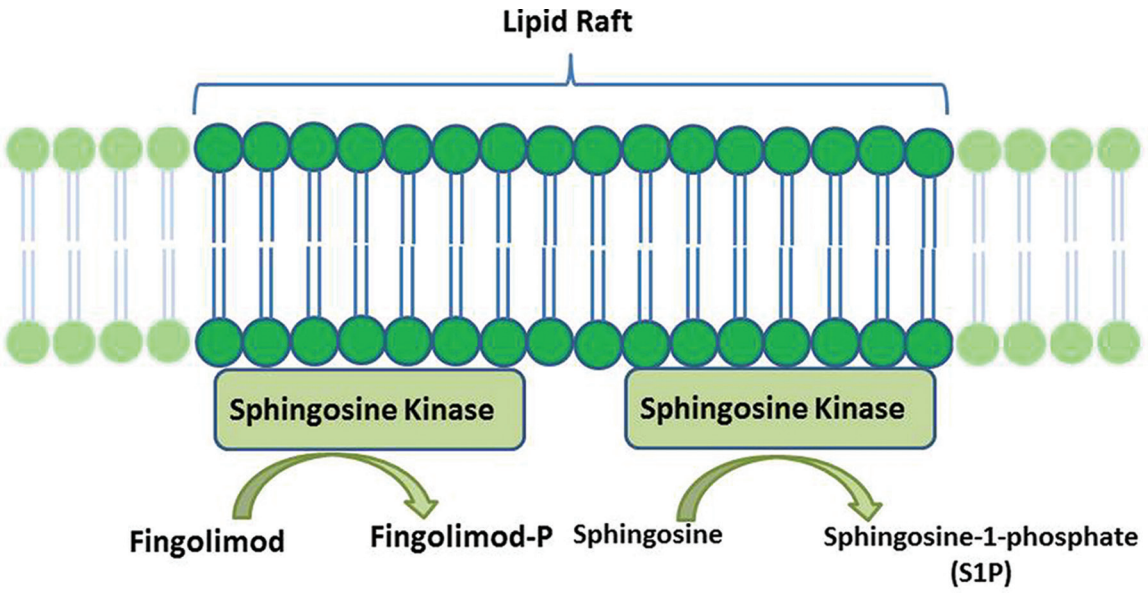

Figure 2. Sphingosine-1-phosphate (S1P) is synthesized in most cells by the actions of sphingosine kinases (SPHK1 and SPHK2), which are localized in lipid raft microdomains. Fingolimod is also phosphorylated by sphingosine kinase to form the active agent, fingolimod phosphate (fingolimod-P), which acts as a S1P receptor modulator, binding with high affinity to four of the five known S1P receptors.

Unfortunately, these trials resulted in the consequence that there is no convincing evidence to support the use of statins as an adjunctive therapy in MS (18).

3.4. Fingolimod (FTY720)

Fingolimod (FTY720), 2-amino-2-propane-1,3-diol hydrochloride, is the first oral disease modifying therapy approved for relapsing forms of MS. Data from clinical trials have indicated that fingolimod is strongly effective for relapse-remitting MS. After the large scale of phase 3 trials, Food and Drug Administration promptly approved fingolimod as a first-line treatment for relapse-remitting MS (19, 20).

Following phosphorylation *in vivo* by sphingosine kinase SPHK2, the active agent, fingolimod phosphate (fingolimod-P), acts as a sphingosine-1-phosphate (S1P) receptor modulator, binding with high affinity to four of the five known S1P receptors (S1P1, S1P3, S1P4 and S1P5) (Figure 2). Fingolimod affects on lymphocyte trafficking, in which the initial activation and eventual downregulation and degradation of S1P1 prevent lymphocyte egress from lymphoid tissues, thereby reducing autoreactive lymphocyte infiltration into the CNS (21). Within this context, fingolimod differentially affects the recirculation of lymphocyte subsets. In MS patients, fingolimod primarily reduced the numbers of $CCR7^{+}CD45RA^{+}$ naive T cells and of $CCR7^{+}CD45RA^{-}$ central memory T cells in blood, whereas $CCR7^{-}CD45RA^{-}$ and $CCR7^{-}CD45RA^{+}$ effector memory T cell subsets remained largely unaffected (21). In addition, fingolimod exerts direct effects on T cell differentiation and function by enhancing the generation and function of regulatory T cells and inhibiting the differentiation of proinflammatory Th1 cells (22, 23, 24). Moreover, because of its lipophilic nature, fingolimod crosses the blood-brain barrier, and might directly downregulate S1P1 and S1P3 in astrocytes. These two receptors have been reported to be upregulated in MS astrocytes (25). Oligodendrocytes and their precursor cells also express S1P receptors particularly S1P5 in mature oligodendorcytes. Neural progenitor cells and some neurons can also express S1P1 along with other S1P receptor subtypes. Microglia can express S1P receptor. The diversity of both cell types and S1P receptor subtypes underscore potential effector activities of fingolimod within the CNS in MS.

S1P is synthesized in most cells by the actions of sphingosine kinases (SPHK1 and SPHK2), which are localized in lipid raft microdomains (Figure 2). Ceramide can be either synthesized *de novo*, originating in the

endoplasmic reticulum, or can be generated at the plasma membrane by hydrolysis of sphingomyelin by neutral sphingomyelinase. Ceramide can be further deacylated by ceramidase to produce D-erythro-sphingosine. Sphingosine is subsequently phosphorylated by SPHK to form S1P. Therefore, inhibition and silencing of SPHK in lipid raft might be a potential new treatment for MS.

3.5. Remyelination promoting antibodies

Rodriguez *et al*. demonstrated a certain population of humoral autoimmune response against CNS antigens is beneficial for CNS remyelination in the Theiler's murine encephalomyelitis virus model of MS (26, 27). They established multiple monoclonal antibodies which promote CNS remyelination and characterized these antibodies (28, 29). The analysis of the antibodies revealed a part of natural autoantibodies, which is encoded by unmutated immunoglobulin germline genes, (30, 31, 32). These antibodies induced CNS myelin repair not only in viral model of MS, but also in autoimmune model (EAE) (33) and toxin (lysolecithin)-induced demyelination (34). They extended their observation and screened a panel of sera from patients with Waldenströme's macroglobulinemia, multiple myeloma, and monoclonal gammopathy of undetermined significance. Consequently, a human IgM antibody was identified (35) and the antibody was sequenced (36). An expression system was engineered to express high titer recombinant this human IgM (rHIgM22)(36). rHIgM22 induced calcium signals in oligodendrocytes *in vitro* and induced myelin repair within demyelinated plaques in Theiler's virus model of MS (36). Its signaling was disrupted by cholesterol depletion, suggesting that the target of rHIgM22 is associated with lipid rafts (37). Subsequent analysis revealed that rHIgM22 is co-localized with integrin β3 associated with lipid rafts but not other integrin β-chains in oligodensrocytes (38). Acorda Therapeutics and the Mayo Foundation are currently conducting phase 1 clinical trial to assess the safety and tolerability of a single dose of rHIgM22 in people with MS (http://www.acorda.com/ Products/rHIgM22.aspx). Additionally, they recently generated another recombinant human IgM antibody, designated rHIgM12, which enhances polarized axonal outgrowth from primary neurons when presented as a substrate *in vitro* and improved motor functions in chronically Theiler's virus-infected SJL mice (39). The epitope of the antibody includes sialic acid because treatment with sialidase disrupted the binding. rHIgM12 bound to neuronal surfaces and induced cholesterol and ganglioside (GM1) clustering, indicating that rHIgM12 functions through a mechanism of axonal membrane stabilization (39). This antibody has a potential to improve neurodegenerative diseases.

4. NEUROMYELITIS OPTOCA (NMO)

Neuromyelitis optica, also known as Devic's disease is an inflammatory disease of the CNS characterized by severe attacks of optic neuritis and transverse myelitis. In 1894, Eugène Devic coined the term "neuromyelite optique aigue" (acute optic neuromyelitis) to describe 16 patients who had lost vision unilaterally or bilaterally and within weeks developed severe transverse myelitis (40). For more than ten decades, there has been long-standing controversy as to whether NMO is a variant of MS or a distinct disease. Wingerchuk *et al*. reported that NMO spinal cord lesions extend over three or more vertebral segments on spinal cord MRI (41). Lennon *et al*. found NMO-IgG, which binds at or near blood brain barrier of mouse brain tissue by indirect immunofluorescent study, serves as a specific marker for NMO (42). Later they demonstrated that NMO-IgG selectively binds to the aquaporin-4 (AQP4) water channel, a component of the dystroglycan protein complex located in astrocytic foot processes at the blood-brain barrier (43). This land-mark discovery has met the controversy to an end and has prompted revisions of the diagnostic criteria for NMO (44).

4.1. Aquaporin-4 (AQP4)

NMO-IgG is considered to be highly specific diagnostic marker for NMO (45, 46, 47) and contributes directly to disease pathogenesis with complement (48, 49). AQP4 is mainly localized on foot processes of astrocytes in the CNS and is expressed in ependymal cells and retinal Müller cells (50). Interestingly, active NMO lesions showed a selective loss of AQP4 immunoreactivity and of glial fibrillary acidic protein (GFAP) containing astrocytes rather than demyelination (51).

The two major AQP4 isoforms, M1 and M23, have identical extracellular domain residues, but M1 has 22 more amino acids at the cytoplasmic N-terminus. NMO-IgG recognizes both M1 and M23. The N-terminal 22 amino acids in M1 contain two cysteine residues at positions 13 and 17. Biochemical analysis and metabolic labeling of transfected cells have revealed that the two N-terminal cysteine residues of AQP4 M1 are palmitoylated (52). This observation suggests that AQP4 is localized on

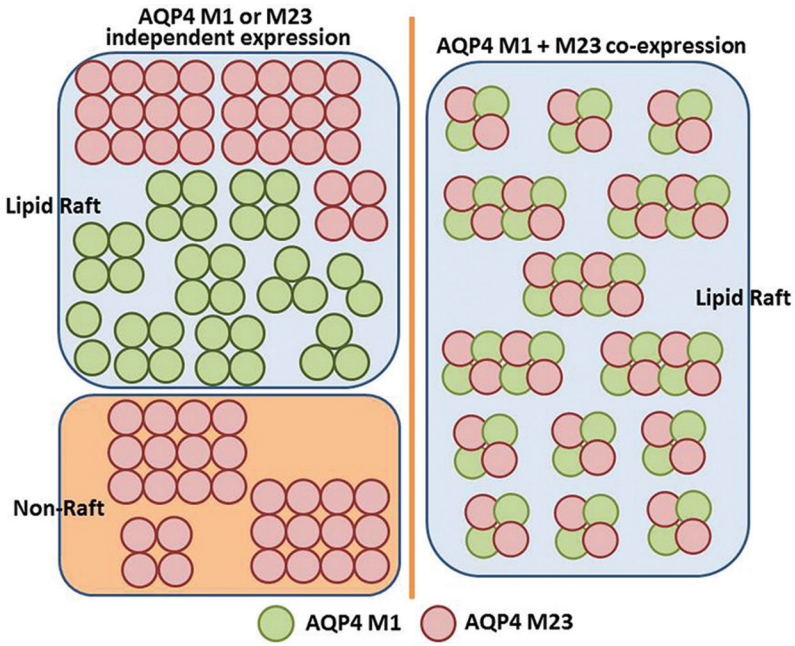

Figure 3. Based upon the sucrose density gradient ultracentrifugation analysis, AQP4 M1 is localized in lipid raft microdomains on the membrane; in contrast, AQP4 M23 is localized in both lipid raft and non-raft fractions when expressed independently (unpublished observation). Interestingly, when both AQP4 M1 and AQP4 M23 are expressed in the same cells, the majority of AQP4 is localized in lipid raft microdomains (unpublished observation).

lipid raft microdomains, because posttranslational modification by palmitoylation on cysteine residue(s) is considered a dynamic targeting mechanism of transmembrane proteins to lipid rafts (53). It has also been reported in primary astrocyte cultures that AQP4 resides in the Triton X-100 insoluble fraction (lipid raft fraction), and that disruption of lipid rafts by depletion of membrane cholesterol with mevastatin, fillipin, or methyl-β-cyclodextrin results in the alteration of AQP4 distribution (54).

Recently we explored the subcellular localization of AQP4 isoforms by generating epitope-tagged AQP4 M1, AQP4 M23, and AQP4 M1/M23 co-expressing cells. The sucrose density gradient ultracentrifugation analysis using an AQP4 M1- or AQP4 M23-expressing cell line revealed that most of AQP4 M1 is localized in lipid raft microdomains on the membrane; in contrast, AQP4 M23 is localized in both lipid raft and non-raft fractions when expressed independently (Figure 3) (unpublished observation). Interestingly, when both AQP4 M1 and AQP4 M23 are expressed in the same cells, the majority of AQP4 is localized in lipid raft microdomains (Figure 3) (unpublished observation).

These previous studies and our recent results strongly suggest that NMO is the disorder of involving membrane lipid rafts of the glial cells in the nervous system. These assumptions would boost the future research areas of this disorder. Moreover, we should explore the new therapeutic methods by coping with the perturbation of lipid rafts functions in this disorder.

4.2. Other aquaporins (AQPs)

The AQPs are a family of small membrane proteins which primary function is thought to facilitate osmotically driven water transport across cell plasma membranes. To date, thirteen mammalian AQPs, AQP0-AQP12, have been identified. Besides AQP4, some of the AQPs have been reported to be associated with lipid rafts.

AQP1 is first identified in 1992 (55). AQP1 is expressed widely in epithelial cells, endothelial cells, and choroid plexus, where it plays an important role in the urinary concentrating system, fluid secretion in the eye and brain, and angiogenesis (56). AQP1 has also been found in fractions containing putative raft-associated molecules such as the ganglioside GM1, sphingomyelin, flottilin, and caveolin. Perturbation

of lipid rafts by cyclodextrin and sphingomyelinase restricted AQP1 diffusion (57).

AQP5 was initially cloned from rat supramandibular glands. AQP5, an apical plasma membrane water channel in salivary glands, lacrimal glands, and airway epithelium, has an important role in fluid secretion. AQP5 localizes in the intracellular lipid rafts under unstimulated conditions, and M3 muscarinic acetylcholine receptor activation by agonist, cevimeline, induce AQP5 trafficking from lipid rafts to nonrafts (58).

AQP0 in lens fiber cells has been extracted in DRMs (59). In the lens the cytoskeletal protein filensin and CP49 interact with AQP0 (Lindsey Rose *et al*., 2006), and interaction with caveolin-1 seems to be involved in the recruitment to DRMs of AQP0 (60). AQP8 and AQP9 of hepatocytes have also been extracted in DRMs (61).

Thus, multiple AQPs have been shown to reside in the DRMs/lipid rafts. The pathophysiological meanings of AQPs and lipid rafts in various diseases should be further elucidated in the future.

5. CONCLUSIONS

Lipid rafts represent a preferential place for lipid–lipid and lipid–protein interactions at the membrane, creating signaling platforms that are involved in numerous neuronal and glial functions. Recent increasing evidences indicate that molecules in the lipid rafts are involved in various neurological disorders including GBS, Alzheimer's disease, Parkinson's disease, and prion disease. As well as these neurological diseases, some of the molecules in lipid rafts are closely related to the pathogenesis or therapeutic targets of MS and NMO. Fingolimod, promising oral immunosuppressive drug for MS, is phosphorylated by sphingosine kinase SPHK2 and acts as a S1P receptor modulator. Therefore, inhibition and silencing of SPHK in lipid raft might be the target of a potential new treatment for MS. The discovery that AQP4 is target antigen of NMO-IgG and anti-AQP4 antibody is a hallmark of NMO, gave an end to the controversy as to whether NMO is a variant of MS or a distinct disease. Our recent observation revealed AQP4 isoforms show distinct subcellular localization. AQP4 M1 is localized in lipid raft microdomains on the membrane; in contrast, AQP4 M23 is localized in both lipid raft and non-raft fractions when expressed independently (unpublished observation). Interestingly, when both AQP4 M1 and AQP4 M23 are expressed in the same cells, the majority of AQP4 is localized in lipid raft microdomains (unpublished observation). Thus, lipid rafts provide us a new aspect to understand the pathomechanism or establishing novel treatment for MS and NMO.

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