Nerve injury associated with complement activation

Pathological studies using human nerve specimens have indicated that complement activation in the nerve membrane is a key process causing nerve damage in GBS [7–10]. Recent *in vitro* and *ex vivo* research on antiganglioside antibodies indicates that complement activation through the classical pathway is essential for pathogenesis of GBS and its variants [11]. In mouse hemi-diaphragma preparations, anti-GQ1b-positive sera showed α-latrotoxin-like effects that increase the frequency of spontaneous release of acetylcholine (ACh) followed by block of elicited ACh release [12]. When normal human serum was used as a source of complement, monoclonal anti-GQ1b IgM antibody also demonstrated similar effects at motor nerve terminals through complement activation, accompanied by considerable deposits of IgM and C3c at nerve terminals [13]. The hypothesis that such nerve injury is induced through complement activation by antiganglioside antibodies is supported by the fact that the IgG subclass of serum anti-GQ1b antibodies from Fisher syndrome (FS) patients is IgG3 or IgG1 that has the ability to fix complement [14]. The complement system is known to be activated via three pathways: classical, lectin and alternative pathways (Figure 1). The classical pathway is initiated by binding of the multimeric collectin, C1q, to Fc portions of immunoglobulin (antibody complexes) bound to the surface antigens in the membrane, followed by the formation of the C3 convertases, C4bC2a and hydrolyzed-C3(C3w)Bb and, finally, leading to the formation of the membrane-attack complex (MAC; C5b-9), opsonization and phagocytosis. The lectin pathway originates by binding of mannose-binding lectin to terminal mannose on bacterial surfaces, followed by the formation of C3 convertase C4bC2a or by the C2 bypass pathway directly producing C3b from C3. The alternative pathway constantly activates at a low level complement cascade resulting in spontaneous hydrolysis of C3 into C3w. This pathway can be amplified easily by various reactions such as the invasion of pathogens. Recent well-conceived studies by Willison's group demonstrated that the

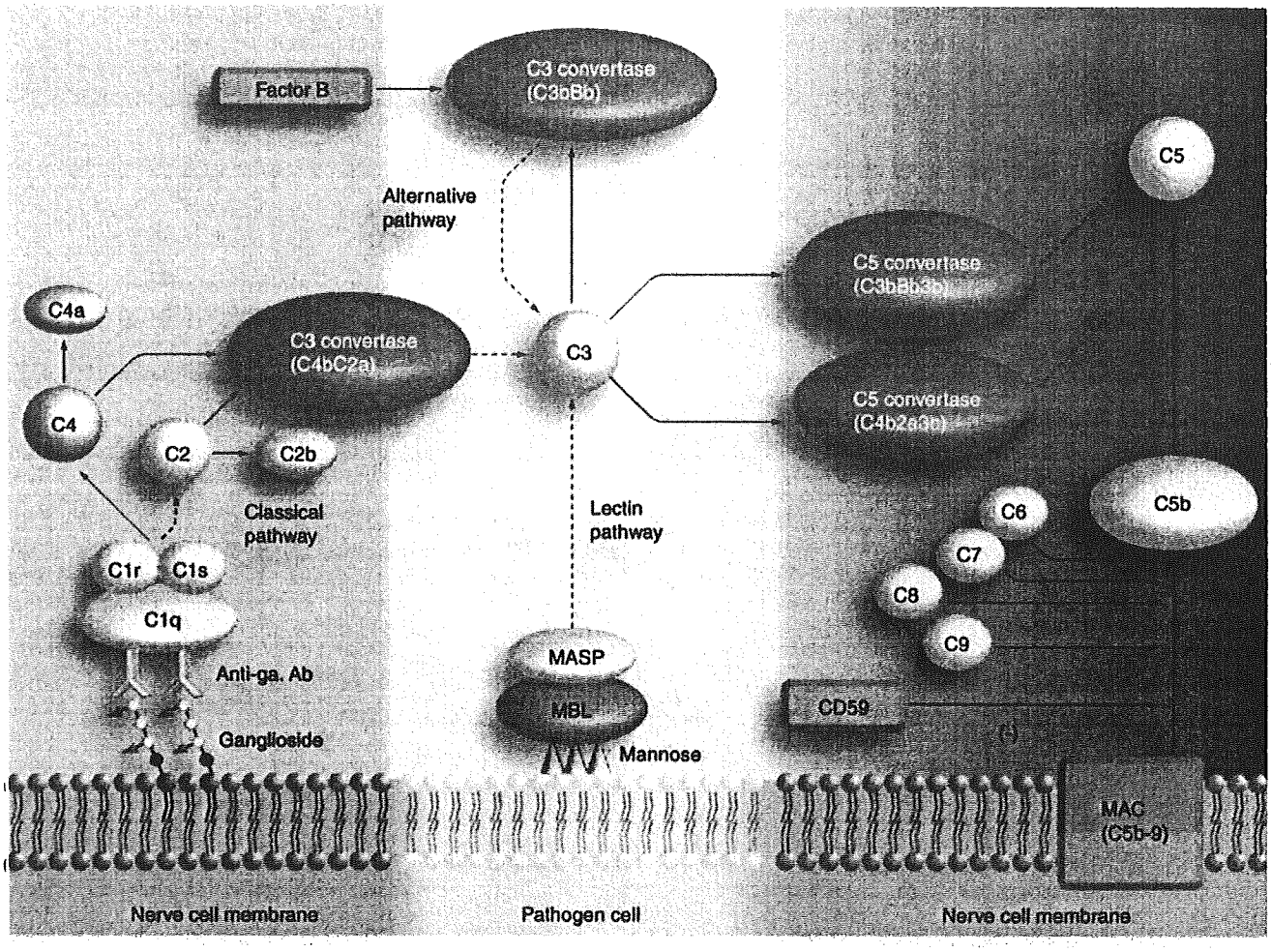


Figure 1. Activation and regulation of the complement system. Shows the potential complement-mediated mechanisms of nerve injury in Guillain–Barré syndrome and its variants. The Lectin pathway can be activated by pathogens of antecedent infection. Anti-ga. Ab: Antiganglioside antibody; MAC: Membrane attack complex; MBL: Mannose-binding lectin; MASP: MBL-associated serine protease.

classical pathway activation with MAC formation is prevailing in the pathophysiology of an experimental model of GBS or FS [15,16]. In their *in vitro* and *in vivo* studies using C6-deficient mice and sera, monoclonal anti-GQ1b IgM antibody did not induce the formation of MAC and increase in MEPP frequency at nerve terminals. In the case of CD59-deficient ($CD59^{-/-}$) mice, which cannot inhibit the formation of MAC, deposits of MAC and damage of perisynaptic Schwann cells and neruofilaments at nerve terminals were more frequently observed than in $CD59^{+/+}$ mice [15]. In a similar experiment using Ca^{2+}-free Ringer, the formation of MAC and loss of neurofilament at nerve terminals were strongly inhibited. This result suggests the predominance of the classical pathway in the aforementioned animal model because the classical pathway is Ca^{2+} dependent and the alternative pathway is Ca^{2+} independent, although there is a possibility that the lectin pathway works together [15].

Disruption of voltage-gated sodium channel clusters at nodes

Immunomodulatory treatments such as plasma exchange (PE) and intravenous administration of immunoglobulin (IVIg) have been developed and utilized in GBS patients, showing a great advantage in that they shorten the period of the inability to walk and improve the prognosis of the disorder. The prompt recovery of clinical features after the immunomodulatory treatments, over hours to days, has often been experienced, indicating that reversible conduction failure or functional block without pathological changes of axon and myelin underlies such prompt recovery of nerve dysfunction. The reversible conduction failure can be induced in acute motor axonal neuropathy (AMAN) that is thought to cause primary axonal damage [17,18]. Impairment of some functional molecule on the nerve membrane is likely to cause the reversible conduction failure or functional block without structural destruction of nerve components. The most potent molecules are ion channels associated with generation of muscle action potentials, especially voltage-gated sodium channels (Nav) that are located and clustered at high densities on the axonal membrane at the nodes of Ranvier. Some studies have indicated that dysfunction of Nav is one of the primary pathophysiological mechanism in GBS [19–21]. In a recent study on axonal excitability in AMAN patients with IgG antibody to GM1, GM1b or GalNAc-GD1a, marked refractoriness (the increase in threshold current during the relative refractory period) was found with a rapid normalization and associated with a recovery of compound muscle action potentials, suggesting that dysfunction of Nav at the nodes of Ranvier is a primary cause of reversible conduction failure in GBS [22•]. Transient blockade of Nav can induce conduction slowing or conduction block and be accompanied by a rapid recovery to normalization within days, as seen in poisoning by the Nav-blocking toxins, saxitoxin and tetrodotoxin [23,24]. Such high refractoriness was not seen in acute inflammatory demyelinating polyneuropathy (AIDP) patients without antiganglioside antibodies [22]. GM1-like epitopes and GalNAc-GD1a are thought to be located in high density at the nodes of Ranvier in motor nerves where Nav clusters are present [25–27•]. Antibody–antigen interaction on the axonal membrane at nodes of Ranvier may induce direct or indirect alteration of regulatory function of Nav. Anti-GM1 antibodies have been shown to induce blockade of Nav at nodes of Ranvier in a complement-mediated manner [19–21,28], although some studies could not confirm anti-GM1 antibody-mediated conduction block or blockade of Nav [29,30].

In the rabbit AMAN model immunized by a bovine brain ganglioside mixture including GM1, reversible dysfunction of Nav with structural changes of the nodes is demonstrated [31]. Lengthened nodes of the ventral roots, disruption of Nav clusters at the nodes, and complement-mediated impairment of paranodal axoglial junctions, nodal cytoskeleton and Schwann cell microvilli are observed in the anti-GM1-positive rabbit model, improving gradually at the late recovery phase [31]. The anti-GM1 monoclonal antibody and the cholera toxin-B subunit stained the membranes of the affected axons in motor nerves of the rabbit, accompanied by deposition of IgG and complement C3. A recent experiment using β1,4-N-acetylgalactosaminyltransferase (GalNAc-T; GM2/GD2 synthase)-knockout ($GalNAcT^{-/-}$) or wild-type mice have shown that GM1 is enriched in the lipid-raft fraction at paranodes and that GM1 plays a role in maintaining the paranodal architecture and clusters of Nav [32]. It is inferred from these findings that the Nav at the nodes of Ranvier in motor nerves is linked to GM1 and that anti-GM1 antibodies may directly cause dysfunction of Nav at the nodes. Taken together, antiganglioside antibody-mediated dysfunction of Nav may be a principal pathogenesis in GBS, especially in AMAN, although other factors such as IL-2 should be considered [33]. In GBS patients demonstrating prompt recovery after immunomodulatory therapies, functional block of Nav without structural destruction of nodes may be a primary mechanism of limb weakness.

Involvement of calcium ion channels in the pathogenesis of GBS

Some studies have suggested that dysfunction of ion channels other than Nav is involved in the pathophysiology of GBS. GalNAc-GD1a, a minor ganglioside in the human brain and peripheral nerves, is a target molecule for serum antibodies in GBS, particularly in a pure motor variant [34–37]. IgG anti-GalNAc-GD1a antibodies from GBS patients or rabbits immunized with GalNAc-GD1a caused complement-independent presynaptic inhibition of ACh release at the neuromuscular junction in rat muscle–spinal cord cocultured cells [38]. The sera from rabbits immunized with GalNAc-GD1a reversibly inhibited voltage-gated Ca channel currents of PC12 pheochromocytoma cells [39•]. These findings suggest that the IgG anti-GalNAc-GD1a antibody blocks neurotransmitter release by its presynaptic inhibitory effect of voltage-gated Ca channel currents through its binding to motor nerve terminals. Sera from AMAN patients with IgG antibodies to GM1, GalNAc-GD1a or GD1a also blocked the Cav2.1 voltage-gated Ca channel current in cerebellar Purkinje cells, but those from AIDP patients did not [40]. Considering that synaptic transmitter release is regulated

by entry of Ca^{2+} via voltage-gated Ca channels at the presynaptic membrane, the possibility exists that antibodies to gangliosides such as GM1 or GalNAc-GD1a block spontaneous muscle action potentials at the neuromuscular junction through impairment of the voltage-gated Ca channel.

In *ex vivo* experiments using mouse hemidiaphragmas, anti-GM1 or anti-GD1a monoclonal antibodies induce a decrease in presynaptic transmitter release in a complement-independent manner through antibody–antigen interaction in the presynaptic membrane of motor nerves, probably because depolarization-induced calcium influx is inhibited [41]. Furthermore, in *in vitro* experiments using cultured olfactory bulb neurons that express P/Q-type Ca channels, these monoclonal antibodies reduced depolarization-induced calcium influx, which was complement-independent [41]. These observations indicate that the complement independent functional blockade of motor nerve terminals by antibodies to GM1 or GD1a can explain limb weakness in AMAN. Considering that the blood–nerve barrier is absent and gangliosides are abundant at presynaptic membranes in the neuro-muscular junction, the presynaptic membranes are likely to be susceptible to antiganglioside antibody attack [42].

Thus, the functional blockade of voltage-gated Ca channels at the presynaptic membrane can be the alternative pathophysio-logy in GBS. Neuromuscular transmission failure, however, has never been confirmed by clinical electrophysiological tests in GBS patients with antibodies to GM1, GD1a or GalNAc-GD1a.

Ganglioside complexes as target antigens in GBS & its variants

There is no question as to the importance of routine measurement for serum antiganglioside antibodies in GBS and its variants. The measurement has generally been done using ELISA system. To exactly detect serum antibodies, we use purified single antigens as test antigens and must avoid contamination of the antigens. Antiganglioside antibody negative has meant so far that the sera have no antibodies to single ganglioside antigens.

Recently, we detected in some GBS and FS sera IgG antibodies to a ganglioside complex (GSC) consisting of two different gangliosides [43,44]. IgG antibodies to the GD1a–GD1b complex (GD1a/GD1b) were identified in eight out of 100 patients with GBS, and their sera showed sharp and strong immunostaining in the overlapping portion of GD1a and GD1b on a thin-layer chromatographic plate (Figure 2) [43]. The anti-GSC antibody-positive sera have little or no reactivity with each constituent ganglioside, but a strong one with a mixture of the two gangliosides in a well of an ELISA plate. This indicates that novel glycoepitopes are formed in the GSC and may function as target molecules in antibody-mediated events. The antibody activity to GD1a/GD1b became maximal when the mixture consisted of an equal amount of GD1a and GD1b. Thus, we cannot state now that sera are antiganglioside antibody negative before antibodies to various GSCs are examined. In a larger population of GBS, 39 out of 234 patients (17%) had IgG antibodies to GSCs consisting of two of the four major ganglio-series gangliosides, GM1, GD1a, GD1b and GT1b . Patients with anti-GD1a/GD1b or anti-GD1b/GT1b antibodies are significantly predisposed to severe disability [45]. Most of anti-GD1a/GD1b- or anti-GD1b/GT1b-positive sera react with GM1/GD1a and GM1/GT1b, indicating that these sera are more multivalent than the antibodies reacting only with GM1/GD1a or GM1/GT1b, or with single ganglioside antigen.

In FS, characterized by a clinical triad of ophthalmoplegia, ataxia and areflexia, a ganglioside GQ1b is considered to be a prime antigen [46,47]. We detected IgG antibodies to GSCs containing GQ1b or GT1a, such as GQ1b/GM1 and GQ1b/GD1a, in half of FS patients [44,48]. FS-associated antibodies are probably subdivided into three types based on antibody specificity: GQ1b-specific, GQ1b/GM1-reactive and GQ1b/GD1a-reactive [44]. Such antibody specificity appears to be regulated by conformation of terminal residues containing sialic acids (Figure 3). That is, anti-GQ1b/GM1-reactive sera react with a combination of (Galβ1–3GalNAc) and (NeuAcα2–8 NeuAcα2–3Galβ1–3GalNAc) in the terminal residues of ganglio-*N*-tetraose structures, and anti-GQ1b/GD1a-reactive sera react with a combination of (NeuAcα2–3Galβ1–3GalNAc) and (NeuAcα2–8 NeuAcα2–3Galβ1–3GalNAc) in the terminal residues [44,48]. A proportion of GBS patients also have IgG antibodies to GSCs containing GQ1b or GT1a, and such anti-GSC antibodies are highly associated with development of ophthalmoplegia in GBS [48]. Both FS patients with and without the anti-GSC antibodies demonstrated the clinical triad, suggesting

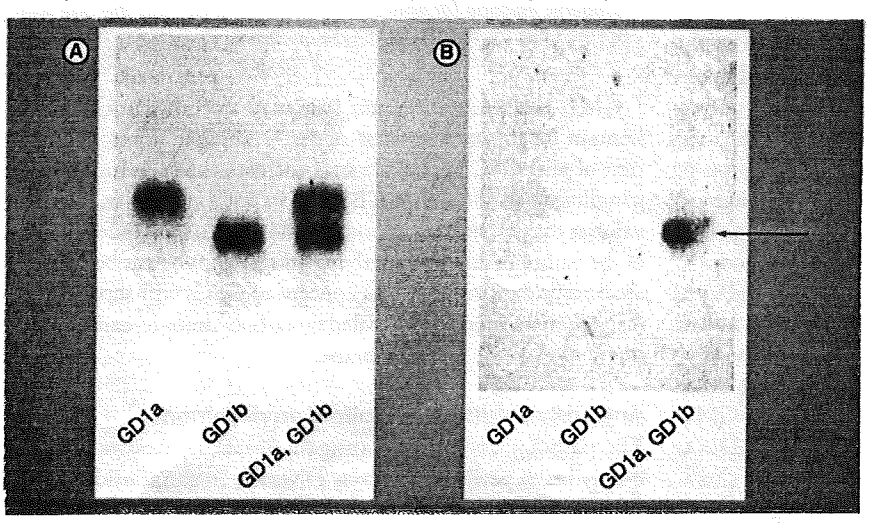


Figure 2. Thin layer chromatography (TLC) study. (A) TLC bands are visualized with orcinol reagent. **(B)** TLC immunostaining study reveals that the overlapping portion between GD1a and GD1b is strongly stained (arrow). Serum is diluted to 1:100.

the possibility that same glycoepitopes are recognized by GQ1b-specific, GQ1b/GM1-reactive, or GQ1b/GD1a-reactive antibody, or that each target molecules are in the vicinity on the nerve membrane. In view of the clustering of gangliosides in the biological membrane, GSCs containing GQ1b appear to be preferential antigens in some cases (Figure 4). As a matter of course, GQ1b must be a key molecule in the immunobiology of FS, and anti-GQ1b IgG antibody remains in place as an excellent diagnostic marker of FS.

The mechanism of anti-GSC antibody-mediated nerve injury remains to be elucidated, although a complement-mediated mechanism is speculated as seen in *ex vivo* or *in vitro* experiments using anti-GQ1b antibody. Ganglioside complexes may be preferentially formed in clustered glycoepitopes in the microdomains, such as lipid rafts or glycosynapses in nerve cell membranes, and anti-GSC antibodies may directly cause dysfunction of nerve cells through binding of anti-GSC antibodies to GSCs in the microdomains. Furthermore, tight binding between such multi-valent anti-GSC antibodies and clustered glycoepitopes may correlate with a predisposition to a severe form of the disease. Alternatively, anti-GSC antibodies may promote the breakdown of the blood–nerve barrier by binding to clustered glycoepitopes in various ligands on the membranes of vascular endothelial cells [49,50]. It has been inferred that ligands of adhesion molecules, such as selectins, comprise diverse and complex glycoconjugates, called clustered saccharide patches, in which oligosaccharides are packed closely together to form rigid rod-like structures with multivalency and strict binding specificity [51,52]. The discovery of anti-GSC antibodies in GBS serum suggests that clustered oligosaccharides on the plasma membrane are actually involved in immune-mediated events. As described in the latest research, clustered glycoepitopes of GSCs in the cell membrane may function in a more effective manner than a solo glycoepitope of isolated ganglioside [53].

Recently, a GSC consisting of GM1 and GalNAc-GD1a (GM1/GalNAc-GD1a) has been reported as a target antigen for pure motor variant of GBS [54]. Electrophysiological findings of the anti-GM1/GalNAc-GD1a-positive patients featured early conduction block at intermediate nerve segments of motor nerves. Serial nerve conduction studies show rapid recovery of the conduction block and no findings indicative of remyelination or axonal degeneration. Hence, the conduction block is thought to be a reversible conduction failure on the axolemma and originates from impairment of axonal membrane properties at the nodes of Ranvier [17,54]. GM1 and GalNAc-GD1a may aggregate and form GM1/GalNAc-GD1a in the motor axonal membrane at nodes, since GM1-like epitopes and GalNAc-GD1a locate on the axolemma at nodes of the motor nerves [26,27]. In view of the dense cluster of of Nav in the axolemma at the nodes, the anti-GM1/GalNAc-GD1a antibody is likely to bind GM1/GalNAc-GD1a at the nodes and cause reversible conduction block through alteration of the regulatory function of Nav. Further investigation will clarify whether anti-GM1/GalNAc-GD1a antibody induces conduction block through complement activation, direct breakdown of Nav function, or both.

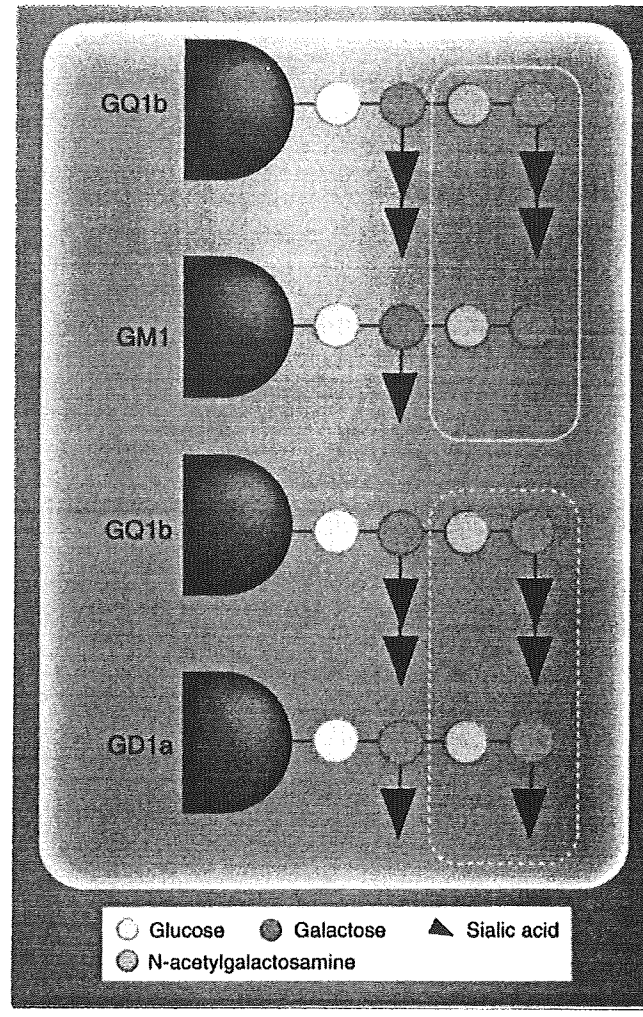


Figure 3. Carbohydrate structures of GQ1b, GM1, and GD1a. The rectangle with solid lines indicates a putative antigenic epitope for anti-GQ1b/GM1 antibody, and the one with dotted lines for anti-GQ1b/GD1a antibody.

GM1, GalNAc-GD1a and Nav may assemble in the microdomain on the axolemma at nodes of Ranvier. Thus, examination of anti-GSC antibodies may increase the spectrum of anti-ganglioside antibodies in GBS and its variants, enhancing their value as diagnostic markers and promoting further understanding of the pathophysiology underlying antiganglioside antibody-mediated nerve dysfunction. The concept of GSCs will shed light on microdomain function mediated by carbohydrate-to-carbohydrate interaction in the cell membrane.

Antibody avidity & glycolipid environment

The pathological effect of antiganglioside antibodies does not evenly reach peripheral nerves. Diversity in ganglioside expression in the PNS can influence development of the symptomatology of GBS with antiganglioside antibodies [6,55], and GBS is subdivided into some clinical phenotypes, each of which is probably associated with specific antiganglioside antibodies [55].

The IgG anti-GQ1b antibody is well known to be an excellent diagnostic marker of FS and have a pathogenetic potential for development of FS [11,46,47]. On the other hand, there are multiple reports showing close association of IgG anti-GD1b antibody with ataxia in GBS and experiments using rabbits sensitized with GD1b also indicate that IgG GD1b-specific antibodies can induce ataxic neuropathy [56,57]. Only half of GBS patients with IgG anti-GD1b antibody, however, demonstrated ataxia in GBS in a larger population of subjects [58]. Considering that different gangliosides can form clusters in living cell membranes [59] and clustered gangliosides such as GSCs are actually involved in antibody-mediated events [43,44], anti-GD1b antibodies may bind to a part of epitopes in the GSCs containing GD1b and differential specificity of the anti-GD1b antibodies may account for the clinical diversity. This hypothesis prompted us to investigate the activities of the anti-GD1b IgG-positive sera against GSCs containing GD1b [60]. We compared antibody activities to GSCs containing GD1b with use of anti-GD1b antibody-positive sera from GBS patients with or without ataxia, demonstrating that anti-GD1b activities to GD1b in sera from patients with ataxia were significantly inhibited by the addition of gangliosides with two or more sialic acids to GD1b. The addition of GD1a to GD1b completely inhibited the binding activity of anti-GD1b antibody to GD1b, suggesting that target epitopes of GD1b can be masked or modified by colocalization of GD1a. These findings indicate that IgG antibodies highly specific for GD1b induce ataxia in GBS and that colocalization of another ganglioside and GD1b may influence the accessibility of the anti-GD1b antibodies (Figure 5) [60]. Finally, the sugar chain of gangliosides may *cis*-interact in the microdomains of the biological membrane and modify the conformation of the glycoepitopes. Such complex glycolipid environments in the cell membrane may affect accessibility and avidity of antiganglioside antibodies against target gangliosides. Regarding IgG anti-GM1 antibodies highly involved in development of pure motor GBS, a recent study using GalNAcT$^{-/-}$ and GD3 synthase-deficient (GD3s$^{-/-}$) mice demonstrated also that the local glycolipid environment in the cell membrane is critical for the exertion of the pathogenetic effect of antiganglioside antibodies [61]. The binding ability of the pathogenetic anti-GM1 antibody to GM1-like epitopes is dependent upon what gangliosides neighbor upon GM1 in the cell membrane and whether GM1-like epitopes are unmasked. Such study has drawn the conclusion that ganglioside interaction may either enable or inhibit antibody binding to the neuronal membrane or be neutral [61]. Thus, to understand the pathogenetic role of antiganglioside antibodies, we should bear in mind that the neuropathic action of antiganglioside antibodies is dependent not only upon the fine specificity of individual antibodies but also upon the conformational diversity of glycoepitopes in glycolipid environments in the biological membrane. GSC antigens can be useful tools for assay of antiganglioside antibody activities against the conformational diversity of the clustered gangliosides in the cell membrane.

Putative targets for AIDP

Acute inflammatory demyelinating polyneuropathy is the most prevalent form of GBS in Western countries, the frequency of which is approximately 90% of total GBS [62]. In addition to cellular immune response associated with activated helper T cells, humoral immune response involved with surface antigens of Schwann cells has been speculated [7,62]. Some glycolipids such as GD1b, LM1 or galactocerebroside have been proposed as target antigens of AIDP [58,63–67]. Experimental allergic neuritis (EAN) induced by inoculation of peripheral myelin protein such as P0 or P2 has been considered as an animal model of AIDP. The predominant pathology of the EAN shows infiltration of T cells and macrophages and demyelination, similar to the histopathology of AIDP [7,68]. Indeed, sera from patients with AIDP often show antibodies to various peripheral nerve myelin, but it is not clear how the antibodies are involved with thr pathophysiology of AIDP [69–71]. The mechanism of conduction failure and demyelination in AIDP has also not been well understood. The disruption of Nav channel clusters at nodes is observed in spinal roots of EAN rats immunized with peripheral myelin, although the mechanism of the disruption was not elucidated [72]. A recent study by Lonigro and Devaux shed light on the disruption of Nav channel clusters at nodes

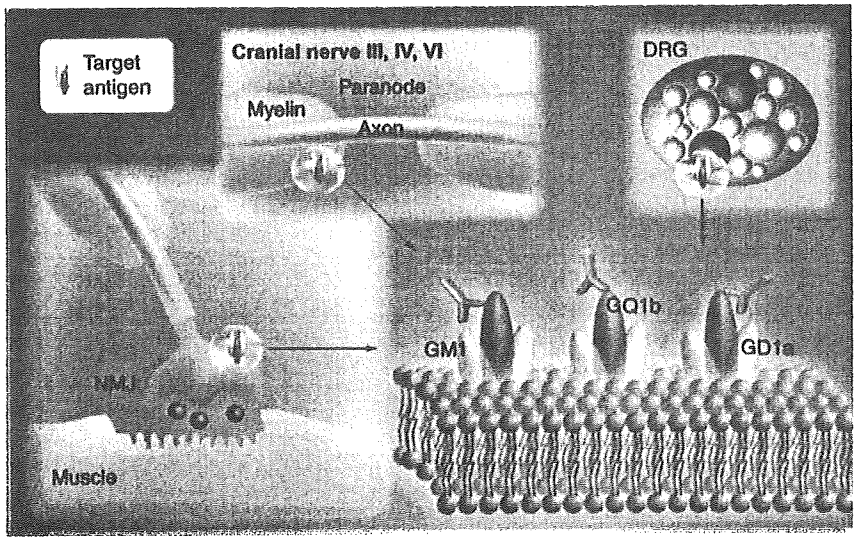


Figure 4. Putative target antigens for autoantibodies in Fisher syndrome. The lower right illustration shows a closeup of the antigenic molecules in the biological membrane. The target antigens are thought to be localized on presynaptic axolemma, perisynaptic Schwann cells, paranodal myelin in oculomotor, trochlear, and abducens nerves, and a subset of large dorsal root ganglion cells. Small balls at neuromuscular junction indicate acetylcholine released from presynaptic axonal membrane.

Table 1. Novel therapeutics for Guillain–Barré syndrome and its variants in preclinical development.

APT070 (Mirococept)	Truncated sCR1 with a membrane-localizing peptide Inhibition of C3/C5 convertase	Mouse model of anti-GQ1b-positive neuropathy	[16]
Eculizumab	Humanized monoclonal antibody against C5 Blocking of the formation of C5a and C5b-9	Mouse model of anti-GQ1b-positive neuropathy	[80]
rEV576	Recombinant form of a complement inhibitory protein derived from the saliva of the soft tick Specific prevention of the conversion of C5 into C5a and C5b	Rat model of myasthenia gravis Mouse model of anti-GQ1b-positive neuropathy	[83,84]
Nafamostat mesilate	Synthetic serine protease inhibitor Inhibition of C1r and C1s, leading to inhibition of C3 fragment deposition	Rabbit model of AMAN	[85]
Flecainide	Sodium channel (Nav 1.5) blocking agent Axonal protection; reduction of axonal degeneration	Rat model of AIDP	[86]
sCR1	Complement regulatory protein Protection of nerves from early axonal degeneration after injury	Rat model of nerve crush injury	[89]

AIDP: Acute inflammatory demyelinating polyneuropathy; AMAN: Acute motor axonal neuropathy; sCR1: Soluble complement receptor 1.

and demyelination in EAN, a model of AIDP [73]. In EAN rats induced by inoculation of crude peripheral myelin, disruption of Nav channel clusters at nodes were found in parallel with the clinical signs, with dispersion of Kv1 channels at nodes and paranodes [73]. Neurofascin 186, a neuronal protein exposed on the surface of axon, and gliomedin, a myelin protein exposed on Schwann cell microvilli, were broken down prior to Nav channel dispersion and demyelination. The early breakdown of neurofascin and gliomedin, which are involved with aggregation of Nav channels at nodes, was followed by the node alteration. Interestingly, antibodies to neurofascin and gliomedin were found in sera from the EAN rats, and associated with degradation of axo–glial units and node alteration. A series of the pathological events at the node were independent from complement deposition, suggesting that antineurofascin and antigliomedin antibodies directly induce the node disorganization without complement activation. EAN rats immunized with synthetic P2 peptide showed little nodal changes and no antibodies to neurofascin and gliomedin with pathology of inflammatory demyelination, indicating that mechanism in EAN induced by immunization of P2 is different from that of EAN by crude peripheral myelin [73]. Thus, the pathophysiology of AIDP are heterogeneous, and humoral factors such as antibodies to neurofascin and gliomedin may also play a crucial role in the pathogenesis of a part of AIDP. From the aspect of treatment, it will be beneficial to know whether the predominant mechanism in a patient with AIDP is humoral or a cellular immune response, dependent upon complement activation or independent. Clinical, electrophysiological and immunobiological studies with a large population of AIDP patients will be required to select more effective treatments or to develop a new regimen.

New therapeutic strategy for GBS & FS

Specific immunomodulatory therapies such as IVIg and PE have hitherto been established in GBS [74]. Plasmapheresis, such as immunoadsorption with tryptophanyl ligands or double membrane filtration, is often utilized as an alternative to PE [75], although a large randomized trial for the efficacy has not yet been executed. Some clinical trials have demonstrated ineffectiveness of corticosteroid in GBS, which might be associated with inhibitory effects of corticosteroids against macrophage repair processes in demyelination [74], and intravenous methylprednisolone (IVMP) therapy executed together with IVIg had no significant benefit for improvement of disability compared with IVIg alone [76]. IVIg plus IVMP may be worthy of reconsideration, because IVIg plus IVMP yielded a better improvement of disability than IVIg alone ($p = 0.06$) [76]. Furthermore, adjustment for age and degree of disability at entry revealed superiority of a combination of IVIg and IVMP ($p = 0.03$) [76]. Such combination therapy might be worthy in a particular subgroup of GBS patients, such as ventilated patients or patients with the severe axonal form.

Ongoing studies on complement-mediated pathophysiology in GBS enable us to challenge novel therapeutic strategies [11] (Table 1). Various complement-targeted drugs are prepared for practical use in many diseases involved in complement activation [77]. A therapeutic inhibitor of complement activation, APT070 (Mirococept, Inflazyme Pharmaceuticals, British Columbia, Canada) contains the C3/C5 convertase inhibiting region of complement receptor 1 and a membrane-localizing peptide. *In vitro* and *in vivo* studies using mouse models of FS demonstrated that APT070 thoroughly prevented MAC (C5b-9) formation and had a neuroprotective effect at the nerve terminal [16]. The humanized monoclonal antibody against complement component C5, eculizumab (Soliris; Alexion Pharmaceuticals Inc., Cheshire, CT, USA), which blocks the formation of human C5a and C5b-9, is the first complement-specific drug authorized by the US FDA, and now applied to treatment of paroxysmal nocturnal hemoglobinuria [78,79]. As with Mirococept, eculizumab also showed neuroprotective potency at motor nerve terminals in a novel mouse model of FS [80]. Intraperitoneal administration of monoclonal anti-GQ1b

Table 2. The target sites of antiganglioside antibodies.

Axonal membrane at nodes of Ranvier	GM1	Yes	[25,26,31]
	GalNAc-GD1a	?	[27]
Presynaptic axonal membrane	GM1	No	[41]
	GD1a	Yes	[41]
		No	[90]
	GalNAc–GD1a	No	[38]
	GQ1b	Yes	[11–13,16]
Perisynaptic Schwann cell	Disialosyl epitope (e.g., GQ1b, GD1b)	?	[15]
Paranodal myelin	GD1b	?	[63]
Paranodal myelin in oculomotor, trochlear, abducens nerves	GQ1b	?	[47]
Subset of large cells	GD1b	? (apoptosis)	[56,87]
	GQ1b	?	[93]

antibody and normal human serum as a complement source induced respiratory paralysis and destruction of nerve terminals in the mouse diaphragm through complement activation. Intravenous eculizumab injection prevented the respiratory paralysis and the complement-mediated nerve injury, which were confirmed immunohistochemically, electrophysiologically and functionally [80]. These results will provide promising therapeutic strategies, but we need take into account some problems. First, it appears to be impossible to administer eculizumab before or at the same time anti-GQ1b antibody attacks peripheral nerves in order to maximize the efficacy. The late administration of eculizumab may fail to inhibit nerve injury that has already begun to progress through complement activation. Second, normal human serum is required as a source of complement in the mouse model of FS. The reason why mouse complement system is not activated by mouse monoclonal anti-GQ1b antibody has not yet been elucidated [11,80]. Considering that it has already been administered to patients with paroxysmal nocturnal hemoglobinuria, a clinical trial of eculizumab will be planned also in GBS and FS patients in near future.

rEV576 is a recombinant form of a complement inhibitory protein identified from the saliva of the soft tick *Ornithodoros moubata*, and inhibits both the classic and the alternative pathways through specific prevention of the conversion of mouse and human C5 into C5a and C5b [81]. Recent reports have described that rEV576 has therapeutic efficacy in animal models of myasthenia gravis in which the terminal stage of complement pathway has been demonstrated to play a key role in pathophysiology [82,83]. In an *in vitro* mouse model of FS using monoclonal anti-GQ1b antibody, rEV576 completely prevented the formation of MAC and protected motor nerve terminals from antibody-mediated nerve injury [84]. Electrophysiological and functional assays revealed the dramatic efficacy of rEV576. This novel agent raises hopes for better-than-expected improvements in the treatment of a subset of GBS and FS in which complement activation plays a pathogenetic role. rEV576 that specifically blocks the C5 activation step is a more attractive agent in that C3b opsonization of pathogens and immune complex solubilization are unaffected by its complement-inhibitory ability.

In a rabbit model of AMAN with anti-GM1 antibodies, a complement inhibitory agent has been shown to inhibit complement deposition and complement-mediated disruption of Nav at the nodes of Ranvier in peripheral motor nerves [85]. A synthetic serine protease inhibitor, Nafamostat mesilate (6-amidino-2-naphtyl-p-guanidinobenzoate dimethanesulfonate), which has been used for acute pancreatitis and disseminated intravenous coagulation, has the complement inhibitory effect and prevents sodium channel disruption in the rabbit model of AMAN [85]. Nafamostat mesilate has often been used for plasmapheresis instead of heparin in Japan, and administered for patients with GBS when they experience plasmapheresis. Such combination of Nafamostat mesilate and plasmapheresis might be more beneficial than plasmapheresis alone. This agent may be a potent candidate for complement inhibitory therapy in light of its affordable price and easy administration.

A sodium channel-blocking agent, Flecainide, which works by blocking the voltage-gated sodium channel Nav1.5 in the heart, has been reported to have an effect on axonal protection in EAN rats sensitized with bovine peripheral myelin [86]. Flecainide ameliorates the neurological deficits, electrophysiological findings indicating demyelination and axonal loss, and axonal damage in tibial nerve fibers in the rat model of AIDP, although the mechanism of its axonal protection has been obscure [86]. This agent may improve the outcome of GBS and its variants, especially AIDP, by protecting the axonal damage.

Expert commentary

Growing evidence supports the importance of complement activation in the pathophysiology of GBS and FS and the neuroprotective effect of complement inhibitory agents, and encourage us to challenge clinical trials of the agents, although some issues of actual use remain to be solved. As is the case of IVIg and PE, its use in the early stage of the disorders should be more beneficial and critical. In the clinical scene, however, complement system has already been activated and nerve injury has progressed to some extent when patients with GBS and FS visit hospitals a few days after the onset of the neurological symptoms. It is unknown whether such late administration of the complement inhibitory agents

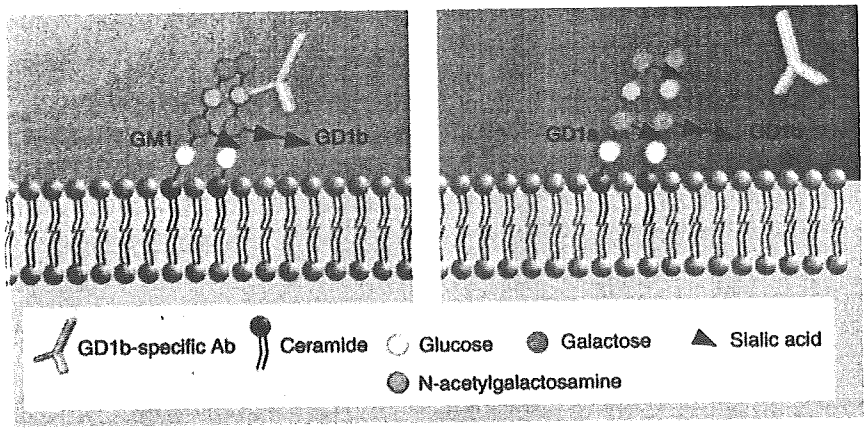


Figure 5. Putative antigen–antibody interaction between GD1b-specific antibody and ganglioside complexes containing GD1b in the nerve cell membrane. In **(A)**, GD1b and GM1 co-localize and *cis*-interact together in the membrane. The GD1b-specific antibody can easily access antigenic epitopes of GD1b in the GD1b-GM1 complex. In **(B)** membrane, GD1b and GD1a co-localize and *cis*-interact together in the membrane. The GD1b-specific antibody can hardly access the antigenic epitopes of GD1b in the GD1b-GD1a complex.

improves the disability and the outcome significantly. Considering that IVIg and PE are more effective if conducted within 2 weeks after the onset of the diseases, the complement inhibitory agents appear to have a potency to limit nerve injury until pathogenetic autoantibodies disappear from the patient's serum. Furthermore, in addition to cost–effectiveness, when or how to administer the complement inhibitory agents should be carefully determined in the practical use, because GBS is a monophasic disorder different from chronic relapsing complement-mediated diseases for which complement inhibitory agents are considered.

The pathophysiology of GBS appears to be heterogeneous. A recent study using a rabbit model of anti-GD1b antibody-associated sensory ataxic neuropathy demonstrated that an apoptotic mechanism is predominant in the pathophysiology of the rabbit model [87]. This suggests that the activation of apoptotic cascade plays a key role in development of anti-GD1b-positive GBS with ataxia, and the complement inhibition may exert little efficacy of nerve protection in the disorder. The efficacy of the complement inhibition has been shown exclusively in a rabbit model of AMAN or a mouse model of FS in which target antigens such as GM1 or GQ1b are shown to be located on the axonal membrane of motor nerves [11,85]. Cellular immune response to myelin antigens, including macrophage activation, can be an alternative mechanism in AIDP, in addition to the complement activation and the formation of the MAC in peripheral nerves [62]. Complement inhibition may be more effective in AIDP in which complement-mediated pathophysiology is predominant. The therapeutic strategy of GBS and its variants should depend on the individual immunobiological mechanism; whether the activation of complement system is predominant or not as well as whether the disorders are electrophysiologically AIDP or AMAN.

Besides blockade of antibody-mediated complement activation, complement inhibition has the therapeutic potential to rescue axons from Wallerian degeneration in impaired nerves. Acute peripheral nerve injury can activate the classical pathway of the complement system and induce the formation of the MAC, which is essential for the early events of axonal degradation during Wallerian degeneration [88]. Moreover, complement inhibition can suppress the recruitment and activation of macrophages [88]. Complement inhibition by soluble complement receptor 1, an inhibitor of all complement pathways, can shield peripheral nerves from early axonal loss [89]. These observations indicate that complement inhibition has a therapeutic potency to directly preclude axonal damage and indirectly inhibit macrophage accumulation in impaired nerves after acute nerve injury, leading to an improved outcome of GBS. Thus, we can expect the protective effect of the complement inhibitory agents against primary or secondary axonal damage in autoimmune neuropathy, although it may be supplemental.

As with of GBS patients demonstrating rapid recovery after immunomodulatory therapies, reversible conduction failure with little pathological changes of nerve structure may cause limb weakness. Antibody-mediated dysfunction of Nav in the axonal membrane at the nodes of Ranvier is probably a cause of the functional block without pathological changes. Interaction between gangliosides and Nav at nodes of Ranvier may contribute to development of such antibody-mediated functional block, which is one of the issues to be solved.

Since the discovery of antiganglioside antibodies in sera from GBS or FS patients, clinical and immunobiological studies on the pathophysiology of GBS has greatly progressed. Close association of anti-GQ1b antibodies with ophthalmoplegia is principally founded on the specific localization of GQ1b on the paranodal myelin in human oculomotor, trochlear and abducens nerves [47]. The antibodies highly specific to GD1b contribute to ataxia in GBS and induce experimental ataxic neuropathy in rabbits immunized with GD1b [60], associated with specific location of GD1b in subsets of large dorsal root ganglion cells [56,57]. Thus, antiganglioside antibody-mediated nerve dysfunction is fundamentally regulated by antibody specificity and specific distribution of target gangliosides in the PNS (Table 2) [6,55]. Recent studies, meanwhile, indicate that some specific conditions of glycoepitopes in the cell membrane of peripheral nerves are required for induction of pathogenetic action of antiganglioside antibodies. First, as mentioned previously, complex glycolipid environments in the cell membrane may affect accessibility and avidity of antiganglioside antibodies against target gangliosides [60,61]. Screening for antibodies to GSCs is essential for elucidating the pathogenetic role of antiganglioside antibodies in the development of GBS and its variants. Analysis of the reactivity of antiganglioside antibodies against GSC antigens is also important. Second, the large amount of targeted ganglioside in specific loci of peripheral nerves makes the loci predisposed to antibody-mediated injury. Anti-GD1a-mediated injury was found

in GD3-synthase knockout mice that overexpressed GD1a but not found in normal mice, suggesting that the high level of expression of GD1a at neuromuscular junctions is required for development of anti-GD1a-mediated disorders [90]. Third, the steric microstructure of gangliosides can influence the binding ability of antiganglioside antibodies. In immunohistochemical experiments using GD1a derivatives which sialic-acid residues were chemically modified, anti-GD1a monoclonal antibodies that preferentially stained motor axons specifically reacted with such GD1a derivatives as GD1a-1-ethyl methyl, GD1a-1-alcohol and GD1a-1-metylester, different from reaction of another anti-GD1a monoclonal antibodies that stained both motor and sensory axons [91]. Anti-GD1a antibodies from AMAN patients revealed the similar reaction to the motor-specific anti-GD1a monoclonal antibodies. Both the fine specificity and ganglioside exposure in the nerves are probably significant contributions to target recognition by antiganglioside antibodies [91].

Various functional molecules that are located on nodes or paranodes and involved in conduction property of motor nerves may be identified in the future. Thrombin receptor PAR-1 (G-protein-coupled protease-activated receptor) is predominantly localized on noncompacted Schwann cell microvilli at the nodes of Ranvier, and interaction of thrombin with the PAR-1 is likely to produce a reversible conduction block in peripheral nerves [92]. Antiganglioside antibody–antigen interaction at nodes may alter the function of molecules such as PAR-1, inducing conduction failure at nodes and muscle weakness in GBS.

Five-year view

Many autoimmune, inflammatory, and infectious diseases have been demonstrated to be attributable to excessive complement activity. Complement-targeted therapeutics is emerging as a hopeful strategy and a salvor to such refractory complement-mediated diseases [77]. There are much data indicating that complement activation underlies development of GBS and its variants. In addition to authorized complement-specific drugs, such as eculizumab, various drug candidates that are in clinical trials and preclinical development will be used to verify their efficacy in *in vitro*, *ex vivo* or *in vivo* models of autoimmune neuropathy, and in the near future will be applied to clinical trials for GBS and its variants. Combination therapy such as complement inhibitory agents and IVIg or PE may be challenged in these trials. To adequately apply novel drugs to patients with GBS and its variants, it is important to understand the mechanisms underlying the disorders in individual cases. Complement-dependent pathophysiology is probably a key role in the development of GBS, and complement-independent mechanisms such as the functional blockade of voltage-gated Ca channel, the apoptotic mechanism, or antineurofascin antibody-mediated disorganization of nodes is not negligible. Precise identification of target epitopes and analysis of their conformation will lead to the development of various efficacious remedies such as anti-idiotypic antibody neutralizing antiganglioside or antineurofascin antibodies. In the near future, indication of novel drugs, the timing of administration, and a various combinations of established treatments and novel drugs should be examined before clinical trials. Combination therapies such as IVIg and a complement inhibitory agent, in practice, will be more feasible than single administration of the novel agent.

Financial & competing interests disclosure

This work was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grants-in-Aid for Scientific Research, 21390273) and the Ministry of Health, Labor and Welfare of Japan (Research Grant for Neuroimmunological Diseases, H20-016, and Health Sciences Research Grant on Psychiatric and Neurological Diseases and Mental Health, H21-012). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Key issues

- In a rabbit or mouse model of Guillain–Barré syndrome (GBS) or Fisher syndrome (FS), complement activation through the classical pathway is essential for development of the disorders.
- In anti-GM1-positive acute motor axonal neuropathy, motor conduction failure is probably a result of antibody-mediated disruption of voltage-gated Na channel clusters at the nodes of Ranvier.
- IgG antibodies to GM1, GD1a or GalNAc-GD1a can induce complement-independent blockade of voltage-gated Ca channel at the presynaptic membrane, explaining a part of the paralysis.
- Ganglioside complexes (GSCs) consisting of two different gangliosides work as target antigens in a proportion of GBS and a half of FS patients.
- Anti-GSC antibodies in GBS sera are real examples indicating that clustered oligosaccharides on the plasma membrane are actually involved in immune-mediated events. Screening for anti-GSC antibodies will broaden the spectrum of antiganglioside antibodies, enhancing their value as diagnostic markers.
- Antiganglioside antibody-mediated nerve dysfunction is fundamentally regulated by antibody specificity and specific distribution of target gangliosides in the PNS.
- The exertion of the pathogenetic effect of antiganglioside antibodies depends upon local glycolipid environment in the cell membrane.
- In acute inflammatory demyelinating polyneuropathy, neurofascin 186 and gliomedin are candidates for target molecules, associated with complement-independent node disorganization.
- In experimental conditions of GBS and its variants, complement inhibitory agents exert a neuroprotective effect by inhibiting activation of the classical pathway.

References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1 Hakomori S. Cell adhesion/recognition and signal transduction through glycosphingolipid microdomain. *Glycoconj. J.* 17(3–4), 143–151 (2000).

2 Simons K, Toomre D. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell. Biol.* 1(1), 31–39 (2000).

3 Todeschini AR, Hakomori SI. Functional role of glycosphingolipids and gangliosides in control of cell adhesion, motility, and growth, through glycosynaptic microdomains. *Biochim. Biophys. Acta* 1780(3), 421–433 (2008).

• Focuses on glycosynaptic microdomains and carbohydrate–carbohydrate interaction in the cell membrane.

4 Simons K, Ehehalt R. Cholesterol, lipid rafts, and disease. *J. Clin. Invest.* 110(5), 597–603 (2002).

5 Kusunoki S, Iwamori M, Chiba A, Hitoshi S, Arita M, Kanazawa I. GM1b is a new member of antigen for serum antibody in Guillain–Barré syndrome. *Neurology* 47(1), 237–242 (1996).

6 Willison HJ, Yuki N. Peripheral neuropathies and anti-glycolipid antibodies. *Brain* 125(12), 2591–2625 (2002).

• Describes the pathogenetic roles of antiganglioside antibodies in the development of autoimmune neuropathy.

7 Hafer-Macko CE, Sheikh KA, Li CY *et al.* Immune attack on the schwann cell surface in acute inflammatory demyelinating polyneuropathy. *Ann. Neurol.* 39(5), 625–635 (1996).

8 Lu JL, Sheikh KA, Wu HS *et al.* Physiologic-pathologic correlation in Guillain–Barré syndrome in children. *Neurology* 54(1), 33–39 (2000).

9 Putzu GA, Figarella-Branger D, Bouvier-Labit C, Liprandi A, Bianco N, Pellissier JF. Immunohistochemical localization of cytokines, C5b-9 and ICAM-1 in peripheral nerve of Guillain–Barré syndrome. *J. Neurol. Sci.* 174(1), 16–21 (2000).

10 Wanschitz J, Maier H, Lassmann H, Budka H, Berger T. Distinct time pattern of complement activation and cytotoxic T cell response in Guillain–Barré syndrome. *Brain* 126(9), 2034–2042 (2003).

11 Willison HJ, Halstead, SK, Beveridge E *et al.* The role of complement and complement regulators in mediating motor nerve terminal injury in murine models of Guillain–Barré syndrome. *J. Neuroimmunol.* 201–202, 172–182 (2008).

• Focuses on complement-dependent pathophysiology in experimental models of Guillain–Barré syndrome and Fisher syndrome.

12 Plomp JJ, Molenaar PC, O'Hanlon GM *et al.* Miller Fisher anti-GQ1b antibodies: α-latrotoxin-like effects on motor end plates. *Ann. Neurol.* 45(2), 189–199 (1999).

13 Goodyear CS, O'Hanlon GM, Plomp JJ *et al.* Monoclonal antibodies raised against Guillain–Barré syndrome-associated Campylobacter jejuni lipopolysaccharides react with neuronal gangliosides and paralyze muscle-nerve preparations. *J. Clin. Invest.* 104(6), 697–708 (1999).

14 Willison HJ, Veitch J. Immunoglobulin subclass distribution and binding characteristics of anti-GQ1b antibodies in Miller Fisher syndrome. *J. Neuroimmunol.* 50(2), 159–165 (1994).

15 Halstead SK, O'Hanlon GM, Humphreys PD *et al.* Anti-disialoside antibodies kill perisynaptic Schwann cells and damage motor nerve terminals via membrane attack complex in a murine model of neuropathy. *Brain* 127(9), 2109–2123 (2004).

16 Halstead SK, Humphreys PD, Goodfellow JA, Wagner ER, Smith RA, Willison HJ. Complement inhibition abrogates nerve terminal injury in Miller Fisher syndrome. *Ann. Neurol.* 58(2), 203–210 (2005).

17 Kuwabara S, Yuki N, Koga M *et al.* IgG anti-GM1 antibody is associated with reversible conduction failure and axonal degeneration in Guillain–Barré syndrome. *Ann. Neurol.* 44(2), 202–208 (1998).

18 Kuwabara S, Asahina M, Koga M, Mori M, Yuki N, Hattori T. Two patterns of clinical recovery in Guillain–Barré syndrome with IgG anti-GM1 antibody. *Neurology* 51(6), 1656–1660 (1998).

19 Arasaki K, Kusunoki S, Kudo N, Kanazawa I. Acute conduction block *in vitro* following exposure to antiganglioside sera. *Muscle Nerve* 16(6), 587–593 (1993).

20 Takigawa T, Yasuda H, Kikkawa R, Shigeta Y, Saida T, Kitasato H. Antibodies against GM1 ganglioside affect K^+ and Na^+ currents in isolated rat myelinated nerve fibers. *Ann. Neurol.* 37(4), 436–442 (1995).

21 Weber F, Rudel R, Aulkemeyer P, Brinkmeier H. Anti-GM1 antibodies can block neuronal voltage-gated sodium channels. *Muscle Nerve* 23(9), 1414–1420 (2000).

22 Kuwabara S, Ogawara K, Sung JY *et al.* Differences in membrane properties of axonal and demyelinating Guillain–Barré syndromes. *Ann. Neurol.* 52(2), 180–187 (2002).

23 Oda K, Araki K, Totoki T, Shibasaki H. Nerve conduction study of human tetrodotoxication. *Neurology* 39(5), 743–745 (1989).

24 Long RR, Sargent JC, Hammer K. Paralytic shellfish poisoning: a case report and serial electrophysiologic observations. *Neurology* 40(8), 1310–1312 (1990).

25 Corbo M, Quattrini A, Latov N, Hays AP. Localization of GM1 and Gal(β 1–3) GalNAc antigenic determinants in peripheral nerve. *Neurology* 43(4), 809–814 (1993).

26 Sheikh KA, Deerinck TJ, Ellisman MH, Griffin JW. The distribution of ganglioside-like moieties in peripheral nerves. *Brain* 122(3), 449–460 (1999).

27 Kaida K, Kusunoki S, Kamakura K, Motoyoshi K, Kanazawa I. GalNAc-GD1a in human peripheral nerve. Target sites of anti-ganglioside antibody. *Neurology* 61(4), 465–470 (2003).

28 Santoro M, Uncini A, Corbo M *et al.* Experimental conduction block induced by serum from a patient with anti-GM1 antibodies. *Ann. Neurol.* 31(4), 385–390 (1992).

29 Hirota N, Kaji R, Bostock H *et al.* The physiological effect of anti-GM1 antibodies on saltatory conduction and transmembrane currents in single motor axons. *Brain* 120(12), 2159–2169 (1997).

30 Dilley A, Gregson NA, Hadden RD, Smith KJ. Effects on axonal conduction of anti-ganglioside sera and sera from patients with Guillain–Barré syndrome. *J. Neuroimmunol.* 139(1–2), 133–140 (2003).

31 Susuki K, Rasband MN, Tohyama K *et al.* Anti-GM1 antibodies cause complement-mediated disruption of sodium channel clusters in peripheral motor nerve fibers. *J. Neurosci.* 27(15), 3956–3967 (2007).

•• Demonstrates that complement-mediated disruption of Nav cluster is the potent mechanism in experimental models of acute motor axonal neuropathy.

32 Susuki K, Baba H, Tohyama K *et al.* Gangliosides contribute to stability of paranodal junctions and ion channel clusters in myelinated nerve fibers. *Glia* 55(7), 746–757 (2007).

• The role of GM1 in the nodal and paranodal structure of peripheral motor nerves.

33 Hartung HP, Reiners K, Schmidt B, Stoll G, Toyka KV. Serum interleukin-2 concentrations in Guillain–Barré syndrome

and chronic idiopathic demyelinating polyradiculoneuropathy: comparison with other neurological diseases of presumed immunopathogenesis. *Ann. Neurol.* 30(1), 48–53 (1991).

34 Kusunoki S, Chiba A, Kon K *et al.* N-acetylgalactosaminyl GD1a is a target molecule for serum antibody in Guillain–Barré syndrome. *Ann. Neurol.* 35(5), 570–576 (1994).

35 Hao Q, Saida T, Yoshino H, Kuroki S, Nukina M, Saida K. Anti-GalNAc-GD1a antibody-associated Guillain–Barré syndrome with a predominantly distal weakness without cranial nerve impairment and sensory disturbance. *Ann. Neurol.* 45(6), 758–768 (1999).

36 Ang CW, Yuki N, Jacobs BC *et al.* Rapidly progressive, predominantly motor Guillain–Barré syndrome with anti-GalNAc-GD1a antibodies. *Neurology* 53(9), 2122–2127 (1999).

37 Kaida K, Kusunoki S, Kamakura K, Motoyoshi K, Kanazawa I. Guillain–Barré syndrome with antibody to a ganglioside, N-acetylgalactosaminyl GD1a. *Brain* 123(1), 116–124 (2000).

38 Taguchi K, Ren J, Utsunomiya I *et al.* Neurophysiological and immunohistochemical studies on Guillain–Barré syndrome with IgG anti-GalNAc-GD1a antibodies-effects on neuromuscular transmission. *J. Neurol. Sci.* 225(1–2), 91–98 (2004).

39 Nakatani Y, Nagaoka T, Hotta S *et al.* IgG anti-GalNAc-GD1a antibody inhibits the voltage-dependent calcium channel currents in PC12 pheochromocytoma cells. *Exp. Neurol.* 204(1), 380–386 (2007).

40 Nakatani Y, Hotta S, Utsunomiya I *et al.* Cav2.1 voltage-dependent Ca^{2+} channel current is ihhibited by serum from select patients with Guillain–Barré syndrome. *Neurochem. Res.* 34(1), 149–157 (2009).

41 Buchwald B, Zhang G, Vogt-Eisele AK *et al.* Anti-ganglioside antibodies alter presynaptic release and calcium influx. *Neurobiol. Dis.* 28(1), 113–121 (2007).

42 Martin PT. Glycobiology of the neuromuscular junction. *J. Neurocytol.* 32(5–8), 915–929 (2003).

43 Kaida K, Morita D, Kanzaki M *et al.* Ganglioside complexes: as new target antigens in Guillain–Barré syndrome. *Ann. Neurol.* 56(4), 567–571 (2004).

•• Study showing that clustered glycoepitopes in the ganglioside mixture are targeted as antigens in Guillain–Barré syndrome.

44 Kaida K, Morita D, Kanzaki M *et al.* Anti-ganglioside complex antibodies in Miller Fisher syndrome. *J. Neurol. Neurosurg. Psychiatry* 77(9), 1043–1046 (2006).

• Study demonstrating that ganglioside complexes containing GQ1b can be target antigens in Fisher syndrome (FS).

45 Kaida K, Morita D, Kanzaki M *et al.* Anti-ganglioside complex antibodies associated with severe disability in GBS. *J. Neuroimmunol.* 182(1–2), 212–218 (2007).

46 Chiba A, Kusunoki S, Shimizu T, Kanazawa I. Serum IgG antibody to ganglioside GQ1b is a possible marker of Miller Fisher syndrome. *Ann. Neurol.* 31(6), 677–679 (1992).

•• Shows close association of IgG anti-GQ1b antibody with FS.

47 Chiba A, Kusunoki S, Obata H, Machinami R, Kanazawa I. Serum anti-GQ1b IgG antibody is associated with ophthalmoplegia in Miller Fisher syndrome and Guillain–Barré syndrome: clinical and immunohistochemical studies. *Neurology* 43(10), 1911–1917 (1993).

• Study immunohistochemically demonstrating localization of GQ1b in human cranial nerves innervating extraocular muscles.

48 Kanzaki M, Kaida KI, Ueda M *et al.* Ganglioside complexes containing GQ1b as targets in Miller Fisher and Guillain–Barré syndromes. *J. Neurol. Neurosurg. Psychiatry* 79(10), 1148–1152 (2008).

49 Kanda T, Yamawaki M, Iwasaki T, Mizusawa H. Glycosphingolipid antibodies and blood–nerve barrier in autoimmune demyelinative neuropathy. *Neurology* 54(7), 1459–1464 (2000).

50 Kanda T, Yamawaki M, Mizusawa H. Sera from Guillain–Barré patients enhance leakage in blood–nerve barrier model. *Neurology* 60(2), 301–306 (2003).

51 Norgard KE, Moore KL, Diaz S *et al.* Characterization of a specific ligand for P-selectin on myeloid cells. A minor glycoprotein with sialylated O-linked oligosaccharides. *J. Biol. Chem.* 268(17), 12764–12774 (1993).

52 Varki A. Selectin ligands. *Proc. Natl Acad. Sci. USA* 91(16), 7390–7397 (1994).

53 Todeschini AR, Dos Santos JN, Handa K, Hakomori S-I. Ganglioside GM2/GM3 complex affixed on silica nanospheres strongly inhibits cell motility through CD82/cMet-mediated pathway. *Proc. Natl Acad. Sci. USA* 105(6), 1925–1930 (2008).

•• Study demonstrating that ganglioside complexes play a functional role in the signal transduction in the cell membrane.

54 Kaida K, Sonoo M, Ogawa G *et al.* GM1/GalNAc-GD1a complex: a target for pure motor Guillain–Barré syndrome. *Neurology* 71(21), 1683–1690 (2008).

55 Kaida K, Ariga T, Yu, RK. Antiganglioside antibodies and their pathophysiological effects on Guillain–Barré syndrome and related disorders: a review *Glycobiology* 19(7), 676–692 (2009).

56 Kusunoki S, Shimizu J, Chiba A, Ugawa Y, Hitoshi S, Kanazawa I. Experimental sensory neuropathy induced by sensitization with ganglioside GD1b. *Ann. Neurol.* 39(4), 424–431 (1996).

•• Milestone paper showing first success in induction of experimental autoimmune neuropathy immunized with a ganglioside antigen.

57 Kusunoki S, Hitoshi S, Kaida K, Arita M, Kanazawa I. Monospecific anti-GD1b IgG is required to induce rabbit ataxic neuropathy. *Ann. Neurol.* 45(3), 400–403 (1999).

58 Miyazaki T, Kusunoki S, Kaida K, Shiina M, Kanazawa I. Guillain–Barré syndrome associated with IgG monospecific to ganglioside GD1b. *Neurology* 56(9), 1227–1229 (2001).

59 Fujita A, Cheng J, Hirakawa M, Furukawa K, Kusunoki S, Fujimoto T. Gangliosides GM1 and GM3 in the living cell membrane form clusters susceptible to cholesterol depletion and chilling. *Mol. Biol. Cell.* 18(6), 2112–2122 (2007).

• Study demonstrating clustering of two gangliosides in the living cell membrane using quick-frozen and freeze-fractured specimens.

60 Kaida K, Kamakura K, Ogawa G *et al.* GD1b-specific antibody induces ataxia in Guillain–Barré syndrome. *Neurology* 71(3), 196–201 (2008).

• Clinical and ***in vitro*** study showing that local glycolipid environment influences the accessibility of antiganglioside antibodies towards target gangliosides.

61 Greenshields KN, Halstead SK, Zitman FM *et al.* The neuropathic potential of anti-GM1 autoantibodies is regulated by the local glycolipid environment in mice. *J. Clin. Invest.* 119(3), 595–610 (2009).

• Experimental study clearly showing local glycolipid environment regulates the pathogenetic effect of antiganglioside antibodies such as anti-GM1 or anti-GD1a antibodies.

62 Hughes RA, Cornblath DR. Guillain–Barré syndrome. *Lancet* 366(9497), 1653–1666 (2005).

63 Kusunoki S, Mashiko H, Mochizuki N *et al.* Binding of antibodies against GM1 and GD1b in human peripheral nerve. *Muscle Nerve* 20(7), 840–845 (1997).

64 Yako K, Kusunoki S, Kanazawa I. Serum antibody against a peripheral nerve myelin ganglioside, LM1, in Guillain–Barré syndrome. *J. Neurol. Sci.* 168(2), 85–89 (1999).

65 Saida T, Saida K, Dorfman SH *et al.* Experimental allergic neuritis induced by sensitization with galactocerebroside. *Science* 204(4397), 1103–1106 (1979).

66 Hahn AF, Feasby TE, Wilkie L, Lovgren D. Antigalactocerebroside antibody increases demyelination in adoptive transfer experimental allergic neuritis. *Muscle Nerve* 16(11), 1174–1180 (1993).

67 Kusunoki S, Shiina M, Kanazawa I. Anti-Gal-C antibodies in GBS subsequent to mycoplasma infection: evidence of molecular mimicry. *Neurology* 57(4), 736–738 (2001).

68 Schmidt B, Toyka KV, Kiefer R, Full J, Hartung HP, Pollard J. Inflammatory infiltrates in sural nerve biopsies in Guillain–Barré syndrome and chronic inflammatory demyelinating neuropathy. *Muscle Nerve* 19(4), 474–487 (1996).

69 Gabriel CM, Gregson NA, Hughes RA. Anti-PMP22 antibodies in patients with inflammatory neuropathy. *J. Neuroimmunol.* 104(2), 139–146 (2000).

70 Kwa MS, van Schaik IN, Brand A, Baas F, Vermeulen M. Investigation of serum response to PMP22, connexin 32 and P(0) in inflammatory neuropathies. *J. Neuroimmunol.* 116(2), 220–225 (2001).

71 Allen D, Giannopoulos K, Gray I. Antibodies to peripheral nerve myelin proteins in chronic inflammatory demyelinating polyradiculoneuropathy. *J. Peripher. Nerv. Syst.* 10(2), 174–180 (2005).

72 Novakovic SD, Levinson SR, Schachner M, Shrager P. Disruption and reorganization of sodium channels in experimental allergic neuritis. *Muscle Nerve* 21(8), 1019–1032 (1998).

73 Lonigro A, Devaux JJ. Disruption of neurofascin and gliomedin at nodes of Ranvier precedes demyelination in experimental allergic neuritis. *Brain* 132(1), 260–273 (2009).

74 Hughes RA, Swan AV, Raphael JC, Annane D, van Koningsveld R, van Doorn PA. Immunotherapy for Guillain–Barré syndrome: a systematic review. *Brain* 130(9), 2245–2257 (2007).

75 Yuki N. Tryptophan-immobilized column adsorbs immunoglobulin G anti-GQ1b antibody from Fisher's syndrome: a new approach to treatment. *Neurology* 46(6), 1644–1651 (1996).

76 van Koningsveld R, Schmitz PI, Meche FG, Visser LH, Meulstee J, van Doorn PA. Effect of methylprednisolone when added to standard treatment with intravenous immunoglobulin for Guillain–Barré syndrome: randomised trial. *Lancet* 363(9404), 192–196 (2004).

77 Ricklin D, Lambris JD. Complement-targeted therapeutics. *Nat. Biotechnol.* 25(11), 1265–1275 (2007).

78 Hillmen P, Young NS, Schubert J *et al.* The complement inhibitor eculizumab in paroxysmal nocturnal hemoglobinuria. *N. Engl. J. Med.* 355(12), 1233–1243 (2006).

79 Rother RP, Rollins SA, Mojcik CF, Brodsky RA, Bell L. Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria. *Nat. Biotechnol.* 25(11), 1256–1264 (2007).

80 Halstead SK, Zitman FM, Humphreys PD *et al.* Eculizumab prevents anti-ganglioside antibody-mediated neuropathy in a murine model. *Brain* 131(5), 1197–1208 (2008).

• The excellent work showing the efficacy of eculizumab in antiganglioside antibody-mediated nerve injury.

81 Fredslund F, Laursen NS, Roversi P *et al.* Structure of and influence of a tick complement inhibitor on human complement component 5. *Nat. Immunol.* 9(7), 753–760 (2008).

82 Hepburn NJ, Williams AS, Nunn MA *et al. In vivo* characterization and therapeutic efficacy of a C5-specific inhibitor from the soft tick *Ornithodoros moubata*. *J. Biol. Chem.* 282(11), 8292–8299 (2007).

83 Soltys J, Kusner LL, Young A *et al.* Novel complement inhibitor limits severity of experimentally myasthenia gravis. *Ann. Neurol.* 65(1), 67–75 (2009).

84 Halstead SK, Humphreys PD, Zitman FM, Hamer J, Plomp JJ, Willison HJ. C5 inhibitor rEV576 protects against neural injury in an in vitro mouse model of Miller Fisher syndrome. *J. Peripher. Nerv. Syst.* 13(3), 228–235 (2008).

85 Phongsisay V, Susuki K, Matsuno K *et al.* Complement inhibitor prevents disruption of sodium channel clusters in a rabbit model of Guillain–Barré syndrome. *J. Neuroimmunol.* 205(1–2), 101–104 (2008).

86 Bechtold DA, Yue X, Evans RM, Davies M, Gregson NA, Smith KJ. Axonal protection in experimental autoimmune neuritis by the sodium channel blocking agent flecainide. *Brain* 128(1), 18–28 (2005).

87 Takada K, Shimizu J, Kusunoki S. Apoptosis of primary sensory neurons in GD1b-induced sensory ataxic neuropathy. *Exp. Neurol.* 209(1), 279–283 (2008).

88 Ramaglia V, King RH, Nourallah M *et al.* The membrane attack complex of the complement system is essential for rapid Wallerian degeneration. *J. Neurosci.* 27(29), 7663–7672 (2007).

89 Ramaglia V, Wolterman R, de Kok M *et al.* Soluble complement receptor 1 protects the peripheral nerve from early axon loss after injury. *Am. J. Pathol.* 172(4), 1043–1052 (2008).

90 Goodfellow JA, Bowes T, Sheikh K *et al.* Overexpression of GD1a ganglioside sensitizes motor nerve terminals to anti-GD1a antibody-mediated injury in a model of acute motor axonal neuropathy. *J. Neurosci.* 25(7), 1620–1628 (2005).

91 Lopez PH, Zhang G, Bianchet MA, Schnaar RL, Sheikh KA. Structural requirements of anti-GD1a antibodies determine their target specificity. *Brain* 131(7), 1926–1939 (2008).

92 Shavit E, Beilin O, Korczyn AD *et al.* Thrombin receptor PAR-1 on myelin at the node of Ranvier: a new anatomy and physiology of conduction block. *Brain* 131(4), 1113–1122 (2008).

93 Kusunoki S, Chiba A, Kanazawa I. Anti-GQ1b IgG antibody is associated with ataxia as well as ophthalmoplegia. *Muscle Nerve* 22(8), 1071–1074 (1999).

Affiliations

• Kenichi Kaida
Division of Neurology, Department of Internal Medicine 3, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama-ken, 359-8513 Japan
Tel.: +81 429 951 617
Fax: +81 429 965 202
adiak901@ndmc.ac.jp

• Susumu Kusunoki
Department of Neurology, Kinki University School of Medicine, 377-372 Ohno-Higashi, Osaka-Sayama, Osaka, 589-8511 Japan
Tel.: +81 72 366 0221
ext. 3553
Fax: +81 72 368 4846
kusunoki-tky@umin.ac.jp

BRAIN RESEARCH 1263 (2009) 10-22

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Transforming growth factor-β1 upregulates keratan sulfate and chondroitin sulfate biosynthesis in microglias after brain injury

Jiarong Yin[a], *Kazuma Sakamoto*[a], *Haoqian Zhang*[a], *Zenya Ito*[a,b], *Shiro Imagama*[a,b], *Satoshi Kishida*[a], *Takamitsu Natori*[a], *Makoto Sawada*[c], *Yukihiro Matsuyama*[b], *Kenji Kadomatsu*[a,*]

[a]*Department of Biochemistry, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan*
[b]*Department of Orthopedics, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan*
[c]*Research Institute of Environmental Medicine, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan*

ARTICLE INFO

Article history:
Accepted 22 January 2009
Available online 3 February 2009

Keywords:
Chondroitin sulfate
Cytokine
Glia
Keratan sulfate

ABSTRACT

After injury to the adult central nervous system, levels of extracellular matrix molecules increase at the injury site and may inhibit the repair of injured axons. Among these molecules, the importance of proteoglycans, particularly their chondroitin sulfate chains, has been highlighted. We have recently reported that keratan sulfate-deficient mice show better axonal regeneration after injury. Here, we investigated the regulation of keratan sulfate and chondroitin sulfate biosynthesis after neuronal injuries. Several key enzymes required for glycosaminoglycan biosynthesis (β3GlcNAcT-7 and GlcNAc6ST-1 for keratan sulfate; CS synthase-1 and C6ST-1 for chondroitin sulfate) were expressed at significantly higher levels in the lesion 7 days after a knife-cut injury was made to the cerebral cortex in adult mice. These increases were accompanied by increased expression of TGF-β_1 and bFGF. Since microglias at the injury sites expressed both keratan sulfate and chondroitin sulfate, the effects of these cytokines were examined in microglias. TGF-β_1 induced the expression of the above-named enzymes in microglias, and consequently induced keratan sulfate and chondroitin sulfate biosynthesis as well as the expression of the chondroitin/keratan sulfate proteoglycan aggrecan in these cells. TGF-β_1 also induced bFGF expression in microglias. bFGF in turn induced TGF-β_1 expression in astrocytes. Astrocyte-conditioned medium following bFGF stimulation indeed induced keratan sulfate and chondroitin sulfate production in microglias. This production was blocked by TGF-β_1-neutralizing antibody. Taken together, our data indicate that the biosyntheses of keratan sulfate and chondroitin sulfate are upregulated in common by TGF-β_1 in microglias after neuronal injuries.

* *Corresponding author.* Fax: +81 52 744 2065.
E-mail address: kkadoma@med.nagoya-u.ac.jp (K. Kadomatsu).

Abbreviations: ACM, astrocyte conditioned medium; bFGF, basic fibroblast growth factor; bFGF-ACM, bFGF-activated astrocyte conditioned medium; CNS, central nervous system; CS, chondroitin sulfate; CSPG, chondroitin sulfate proteoglycan; EGF, epidermal growth factor; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcA, glucuronic acid; GlcNAc, N-acetylglucosamine; GlcNAc6ST-1, N-acetylglucosamine 6-O-sulfotransferase-1; KS, keratan sulfate; KSPG, keratan sulfate proteoglycan; RT-PCR, reverse-transcription polymerase chain reaction; TGF-β_1, transforming growth factor-β_1

0006-8993/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.brainres.2009.01.042

1. Introduction

Injuries in the adult mammalian central nervous system (CNS) often leave serious functional defects, since neuronal axons do not spontaneously regenerate after injuries. Many factors are thought to be involved in this inability of axons to regenerate, and can be summarized mainly into two categories: (1) lack of intrinsic regenerative capacity (Neumann and Woolf, 1999; Widenfalk et al., 2001) and (2) production of inhibitory factors (De Winter et al., 2002; Filbin, 2003; Silver and Miller, 2004). Among the inhibitory factors, myelin-associated molecules, such as Nogo, MAG and Omgp, have been studied extensively, although studies using their knock-out mice or their receptor-deficient mice show that these factors are not sufficient for the *in vivo* inhibition of axonal regeneration (Filbin, 2003; Silver and Miller, 2004). Other chemorepulsive molecules, such as Sema3A and RGMa, also play important roles in inhibiting axonal regeneration (De Winter et al., 2002; Hata et al., 2006; Kaneko et al., 2006).

Besides these molecules, the importance of chondroitin sulfate proteoglycans (CSPGs) has been recently highlighted (Moon et al., 2001; Bradbury et al., 2002; Grimpe and Silver, 2004). Proteoglycans are a group of proteins that link acidic polysaccharides, i.e., sulfated glycosaminoglycans, of which there are three main forms: chondroitin sulfate (CS)/dermatan sulfate, keratan sulfate (KS) and heparan sulfate/heparin (Scott et al., 1990; Johnson-Green et al., 1991). The inhibitory function of CSPGs on axonal outgrowth is largely ascribed to their covalently attached CS-glycosaminoglycans, since the ablation of CS by the use of chondroitinase ABC or a DNA enzyme as to xylosyltransferase enhances neuronal axon growth at the site of CNS injury (Moon et al., 2001; Bradbury

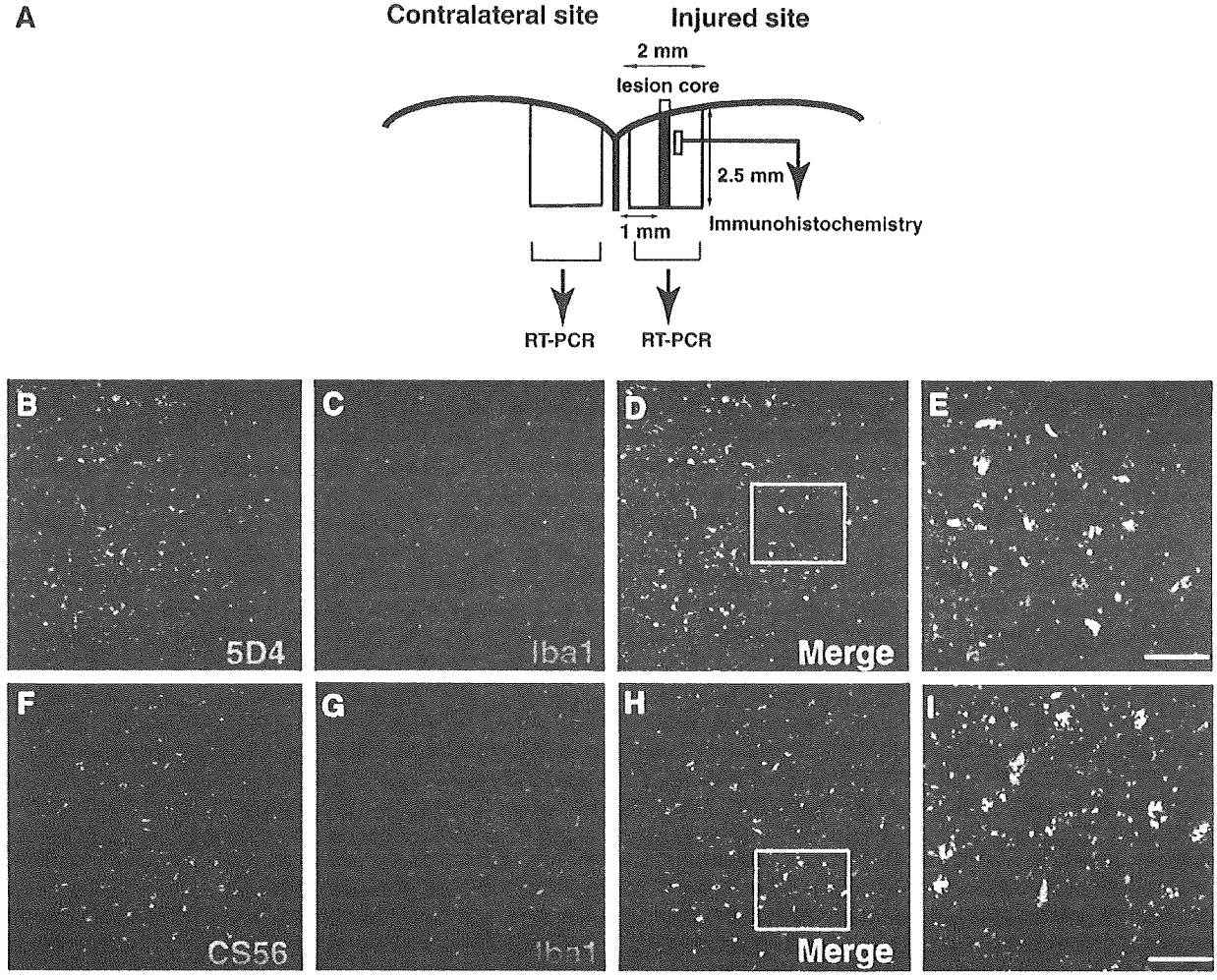


Fig. 1 – Expression of KS and CS by microglia after brain injury. (A) Schematic localization of samples for immunohistochemistry and RT-PCR. (B–E) 5D4-reactive KSPG was expressed by microglia at the site of injury. 5D4 is specifically reactive to KS, and Iba1 specifically recognizes microglias. (F–I) Microglias expressed CSPG at the site of injury. CS56 specifically recognizes CS. The specimens were treated with ChABC before immunohistochemical staining with 5D4 antibody. (E and I) show magnified photos corresponding to the squares in (D and H) respectively. Bar, 20 μm.

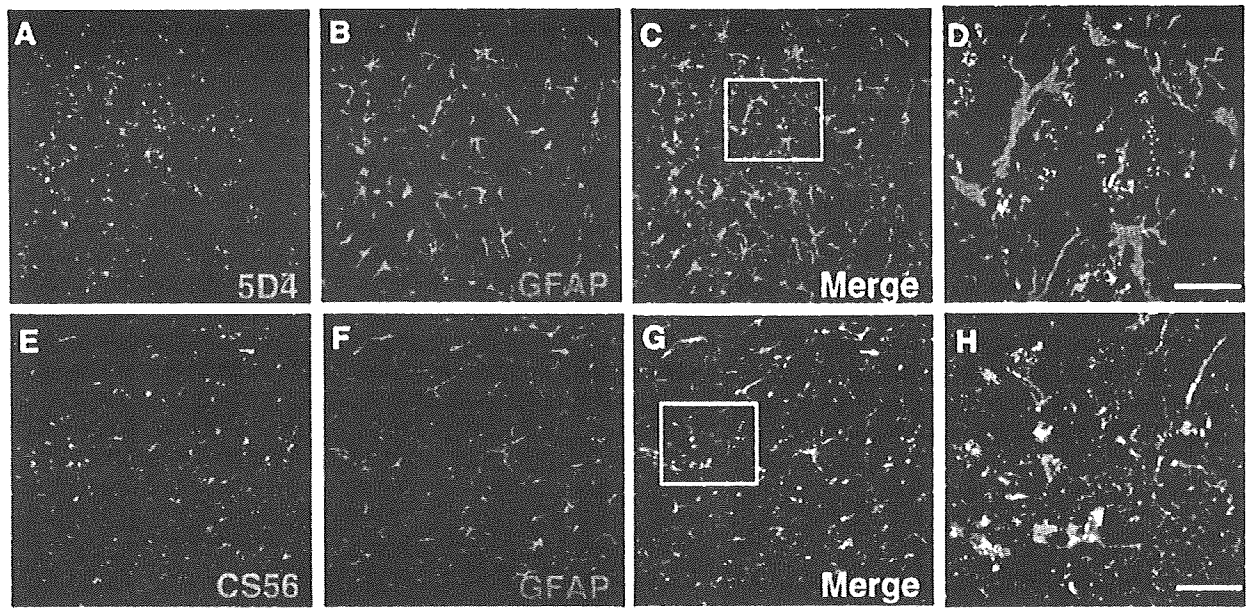


Fig. 2 – Relationships between astrocytes and expression of KS and CS after brain injury. (A–D) 5D4-reactive KSPG only partly merged with astrocytes at the site of injury. (E–H) CS56-reactive CSPG expression merged with astrocytes at the site of injury. The specimens were treated with ChABC before immunohistochemical staining with 5D4 antibody. (D and H) show magnified photos corresponding to squares in (C and G) respectively. Bar, 20 μm.

et al., 2002; Grimpe and Silver, 2004). For example, the axon growth of dopamine neurons is enhanced by chondroitinase ABC treatment after nigrostriatal tract transection (Moon et al., 2001). Chondroitinase ABC treatment has been shown to enhance functional recovery after spinal cord injury in a rat model (Bradbury et al., 2002).

We have recently demonstrated that KS is important in inhibiting axonal regeneration. Thus, after a stab wound to the cerebral cortex, mice deficient in KS of the CNS [N-acetylglucosamine 6-O-sulfotransferase-1 (GlcNAc6ST-1)-deficient mice] show less glial scar formation and enhanced neuronal axon regeneration than do wild-type mice, even though the induction of CS expression is comparable to that in wild-type mice (Zhang et al., 2006). KS ablation ameliorates functional disturbance after spinal cord injury (Imagama et al., unpublished data). These data suggest that KS plays a critical role in inhibiting axonal regeneration.

This background indicates the importance of verifying the mechanisms underlying the induction of KS and CS expression after neuronal injury. It has been recently reported that the inhibition of CS chain polymerization in astrocytes via RNA interference decreases the inhibitory activity of CSPG against neurite outgrowth (Laabs et al., 2007). It was also reported that among various CS units the C unit is upregulated after neuronal injury (Properzi et al., 2005). Some other studies also investigated glycosaminoglycan levels in the central nervous system with epilepsy or after injury (Perosa et al., 2002; Dobbertin et al., 2003). However, detailed analyses involving expression of synthetic enzymes for KS biosynthesis after neuronal injury are still needed. Here, we demonstrate that the biosyntheses of KS and CS share a regulation mechanism in common which is mediated by TGF-β_1 in microglias after neuronal injury.

2. Results

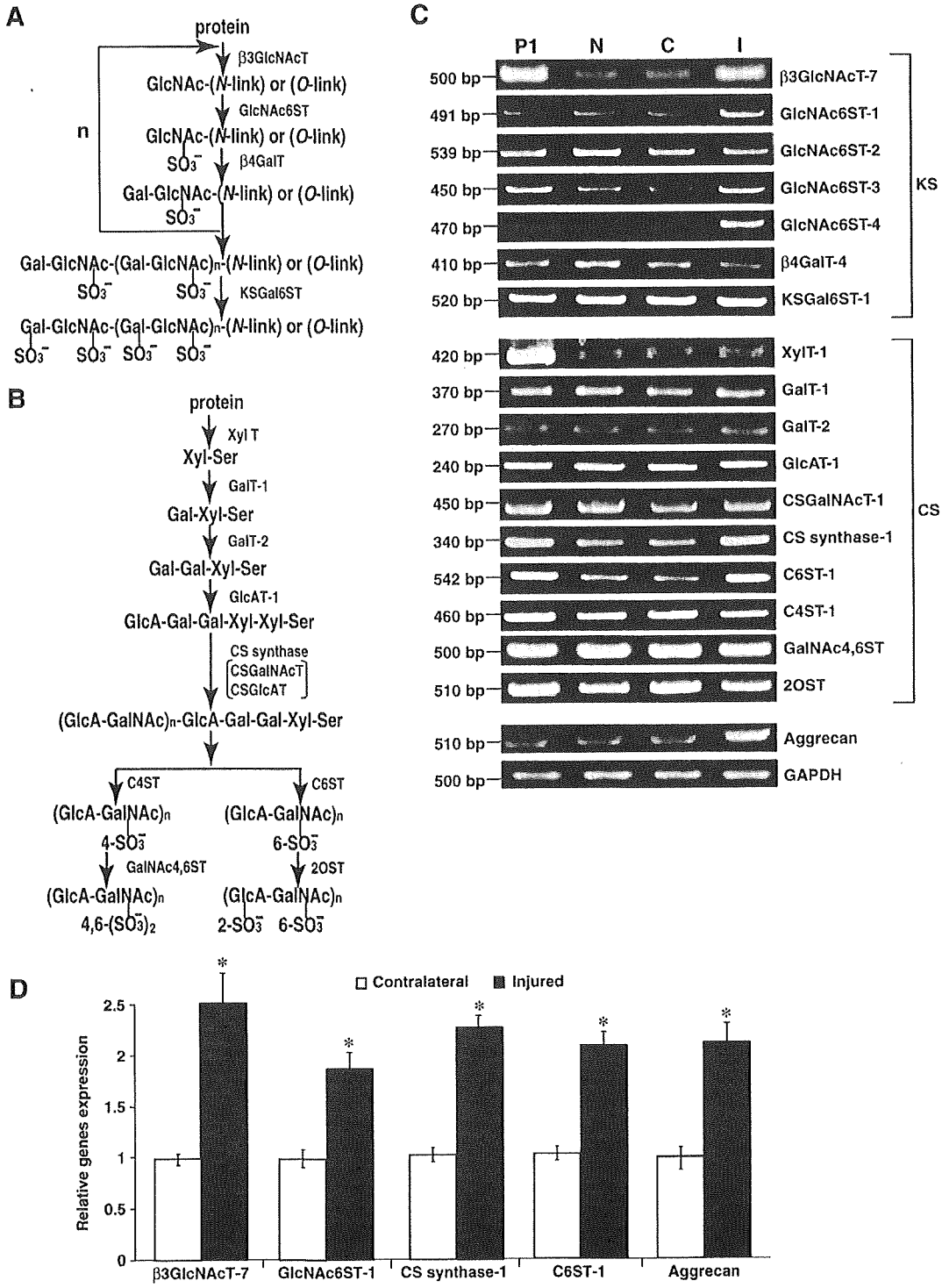
2.1. *Glial cells expressing KS and CS*

We made a knife-cut injury to the right hemisphere of the cerebral cortex in 8-week-old male mice, and localized KS and CS expression in the brain 7 days after injury. 5D4 is an antibody specific to KS, while the antibody CS56 specifically

Fig. 3 – Expression of synthases of KS, CS and aggrecan after brain injury. (A) The biosynthesis process of KS. GlcNAc6ST, N-acetyl-glucosamine 6-O-sulfotransferase; β3GlcNAcT, β1,3 N-acetylglucosaminyltransferase; KSGal6ST, keratan sulfate galactose 6-O-sulfotransferase; β4GalT, β1,4-galactosyltransferase. (B) The CS synthesis process. XylT, xylose transferase; GalT, galactose transferase; GlcAT, glucuronic acid transferase; C6ST, chondroitin 6-O-sulfotransferase; CSGalNAcT, chondroitin sulfate N-acetyl-galactosaminyltransferase; CSGlcAT, chondroitin sulfate glucuronic acid transferase; C4ST, chondroitin 4-O-sulfotransferase; GalNAc4,6ST, N-acetyl-galactosamine-4-sulfate 6-O-sulfotransferase; 2OST, uronosyl 2-O-sulfotransferase. (C) Expression of KS and CS synthases after brain injury. For RNA extraction of injured lesion (I) and contralateral region (C), tissues were excised 7 days after brain injury. Postnatal day 1 brain (P1) and normal adult brain (N) were also subjected to RNA extraction. RNA expression was estimated by RT-PCR. (D) Real-time PCR for the quantification of RNA expression. The results represent means ± SD ($n=3$). *$P<0.05$ (versus contralateral region).

recognizes CS. Regions beside the lesion core were subjected for the immunofluorescence analysis (Fig. 1A). A significant area positive for KS and CS expression merged with microglias that were reactive to antibody Iba1 (Figs. 1B–I). The experiments were performed on two individual mice, and showed similar results.

In contrast to the results of microglias, areas positive for KS expression only partly merged with reactive astrocytes, whereas most astrocytes appeared to express CS (Figs. 2A–H). These results were consistent with those reported for KS expression in rat spinal cord after injury (Jones and Tuszynski, 2002). Although we did not exclude the possibility that a portion of KS is produced by reactive astrocytes, our data suggested that KS and CS production by microglias was a suitable subject for the study of glycosaminoglycan biosynthesis regulation.



2.2. *Expression profiles of enzymes for KS and CS biosynthesis after brain injury*

KS and CS are biosynthesized by many enzymes (Figs. 3A, B). KS is composed of repeating disaccharide units of galactose (Gal) and *N*-acetylglucosamine (GlcNAc), where the C6 position of GlcNAc is always sulfated. The reaction sequence for the biosynthesis of KS consists of *N*-acetylglucosaminylation, 6-sulfation of a GlcNAc residue exposed at the nonreducing end, and galactosylation (Fig. 3A) (Habuchi et al., 2006; Kitayama et al., 2007). CS is composed of glucuronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc). The C2 position of GlcA and the C4 and C6 positions of GalNAc can be sulfated. Xylose is first linked to serine residues, followed by the sequential addition of Gal, Gal and GlcA. The first GalNAc of the CS polymer is then added by a CSGalNAc transferase, and then polymerization (composed of GlcA and GalNAc) is accomplished by CS synthases (Fig. 3B) (Silbert and Sugumaran, 2002; Sakai et al., 2007).

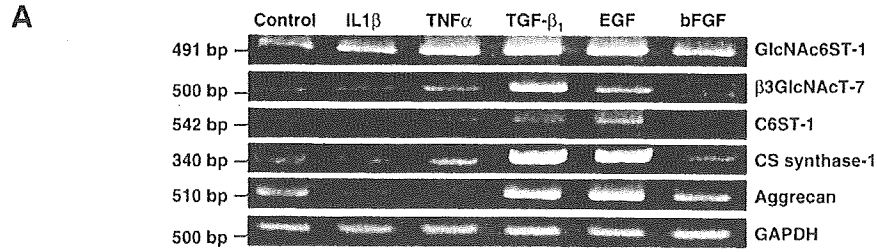
Among enzymes required for KS or CS biosynthesis, we picked up several representative ones as listed in Fig. 3C, so that we could determine at least some of the enzymes upregulated during KS and CS biosynthesis *in vivo*. To obtain expression profiles of these enzymes, tissues from the injury lesion as well as from the contralateral regions ('I' and 'C', respectively, in Fig. 3C) were excised 7 days after a knife-cut injury to the right hemisphere of the cerebral cortex in 8-week-old male mice was made (Fig. 1A). The excised tissues were subjected to RNA extraction and further RT-PCR. RT-PCR was also performed for uninjured 8-week-old male mouse brain ('N') and postnatal day 1 mouse brain ('P1'). In this case, P1 samples were used as a positive control, since KS and CS are known to be highly expressed in P1 mouse brain (Snow et al., 1990; Emerling and Lander, 1996; Miller et al., 1997; Oohira et al., 2000; Zhang et al., 2006). We found that GlcNAc6ST-1, -3, -4 and β3GlcNAcT-7 expression was markedly increased in the injured lesion compared with that in the contralateral region and in uninjured brain (Fig. 3C). The expression of two other enzymes related to KS biosynthesis, e.g., GlcNAc6ST-2 and KSGal6ST, was unchanged (Fig. 3C). As to CS biosynthesis, the expression of CS synthase-1 and C6ST-1 was at least found to be increased in the injured lesion compared with that in the contralateral region and in uninjured brain (Fig. 3C). These results suggest that upregulation of GlcNAc6ST-1, -3, -4 and β3GlcNAcT-7 expression might be involved in the elevation of KS biosynthesis, while upregulated CS synthase-1 and C6ST-1 expression was related to the increase in CS biosynthesis. We further examined the expression of aggrecan as an example of a proteoglycan that carries both KS and CS (Fig. 3C). As shown in Fig. 3C, aggrecan expression also was enhanced by brain injury. We performed RT-PCR for Fig. 3C using 2 sets of samples, and obtained similar results. Data shown in Fig. 3C is a representative one. The expression upregulation observed in Fig. 3C was confirmed by quantitative RT-PCR (Fig. 3D).

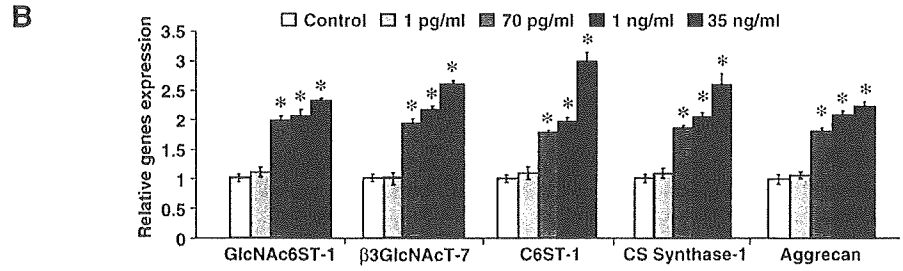
2.3. *KS and CS expression in microglias under the control of cytokines*

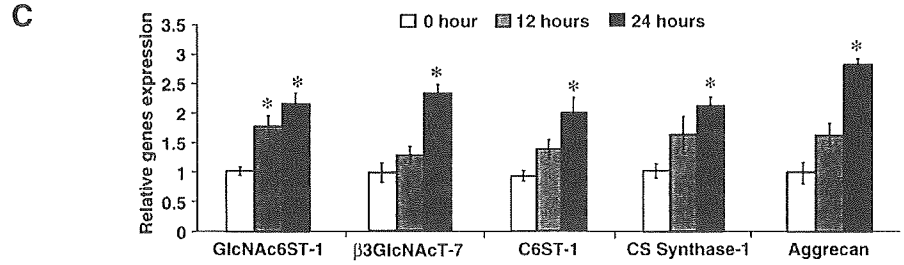
Many cytokines have been implicated in the function of astrocytes and/or microglias (Faber-Elman et al., 1996; Ridet et al., 1997; Schilling et al., 2001). Therefore, we next examined the response of primary cultured microglias to cytokines. Primary cultured microglias were exposed to various cytokines for 24 h, after which the expression of enzymes for KS and CS biosynthesis was examined. The examined enzymes had been upregulated *in vivo* after brain injury (Figs. 3C, D), and are known to be involved in KS and CS biosynthesis (GlcNAc6ST-1 and β3GlcNAcT-7 for KS; C6ST-1 and CS synthase-1 for CS: Figs. 3A, B). In this case, we chose GlcNAc6ST-1 instead of GlcNAc6ST-3 and -4, since our previous study has demonstrated that GlcNAc6ST-1 is essential for inducing KS biosynthesis after neuronal injury (Zhang et al., 2006). Among the cytokines tested, TGF-β_1 and EGF upregulated the expression of GlcNAc6ST-1, β3GlcNAcT-7, C6ST-1 and CS synthase-1 (Fig. 4A). Aggrecan expression was also upregulated only by TGF-β_1 and EGF (Fig. 4A). Quantitative RT-PCR verified that these expressions were indeed upregulated by TGF-β_1 in a dose-dependent manner (Fig. 4B). TGF-β_1 at 70 pg/ml could increase expressions of the enzymes (Fig. 4B). Data in Figs. 4A and B were obtained from microglias exposed to TGF-β_1 for 24 h. We then investigated time course of the expressions. The upregulation of GlcNAc6ST-1 occurred as early as 12 h after TGF-β_1 stimulation, whereas expressions of other molecules showed a tendency of increase at this time point (Fig. 4C).

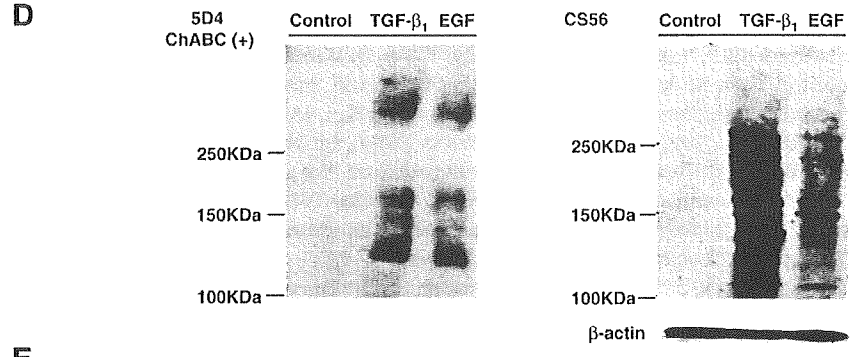
Consistent with these data, TGF-β_1 increased production of KS- and CS-bearing proteoglycans, which appeared as smear bands on Western blot analysis (Fig. 4D). EGF increased the expression of these proteoglycans, but to a lesser extent than TGF-β_1 did (Fig. 4D). Consistent with data shown in Fig. 4B, the KS- and CS-reactivity on Western blot increased in a dose-dependent manner (Fig. 4E). The increase became apparent 12 h after TGF-β_1 stimulation (Fig. 4E). Taking the data in Fig. 4C into account, a post-transcriptional regulation of enzyme

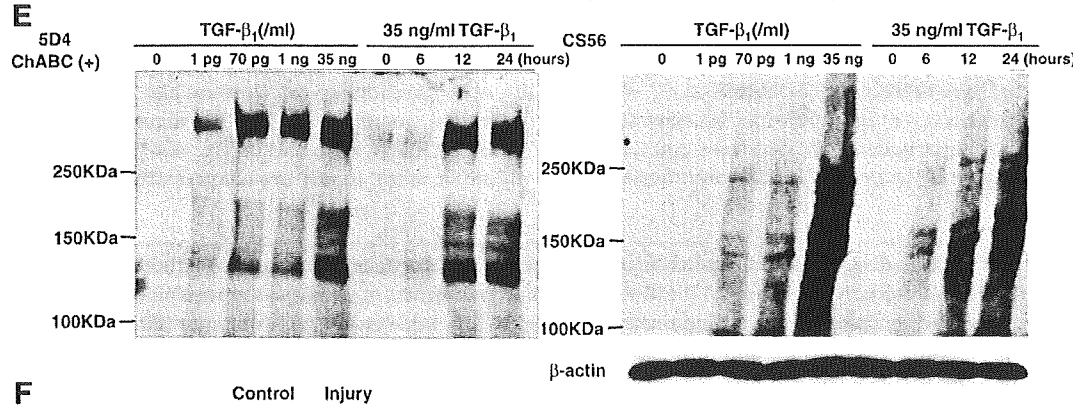
Fig. 4 – Induction of KS, CS and aggrecan expression in primary cultured microglia by cytokines. (A) Primary cultured microglias were stimulated with indicated cytokines for 24 h. RT-PCR was then performed to examine expression of aggrecan and enzymes that increased after brain injury, as demonstrated in Fig. 3C. (B) Real-time PCR for the quantification of RNA expression. The results represent means ± SD ($n=3$). *$P<0.05$ (versus control). Primary cultured microglias were stimulated with indicated cytokines for 24 h. (C) Time course of enzyme expressions for KS and CS biosynthesis. Real-time PCR was performed for the quantification of RNA expression. The results represent means ± SD ($n=3$). *$P<0.05$ (versus 0 hour). (D) The expression of KSPG and CSPG was examined by Western blot analysis. β-actin controls are common for the 5D4 and CS56 Western blots, since the sources of the samples are the same. (E) Dose-dependency and time course of KSPG and CSPG expressions. β-actin controls are common for the 5D4 and CS56 Western blots, since the sources of the samples are the same. (F) RT-PCR showed the induction of TGF-β_1 and bFGF expression after brain injury. Tissues from injured lesion ('Injury') and contralateral regions ('Control') were collected 7 days after brain injury. RNA extracted from the tissues was then subjected to RT-PCR.

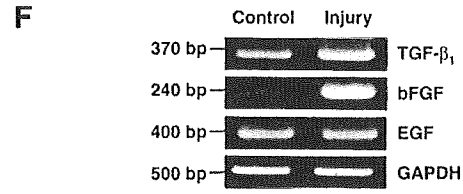

A
Control
IL1β
TNFα
TGF-β1
EGF
bFGF
491 bp
500 bp
542 bp
340 bp
510 bp
500 bp
GlcNAc6ST-1
β3GlcNAcT-7
C6ST-1
CS synthase-1
Aggrecan
GAPDH


B
Control
1 pg/ml
70 pg/ml
1 ng/ml
35 ng/ml
Relative genes expression
GlcNAc6ST-1
β3GlcNAcT-7
C6ST-1
CS Synthase-1
Aggrecan


C
0 hour
12 hours
24 hours
Relative genes expression
GlcNAc6ST-1
β3GlcNAcT-7
C6ST-1
CS Synthase-1
Aggrecan


D
5D4
ChABC (+)
Control
TGF-β1
EGF
250KDa
150KDa
100KDa
CS56
Control
TGF-β1
EGF
250KDa
150KDa
100KDa
β-actin


E
5D4
ChABC (+)
TGF-β1(/ml)
0
1 pg
70 pg
1 ng
35 ng
35 ng/ml TGF-β1
0
6
12
24 (hours)
250KDa
150KDa
100KDa
CS56
TGF-β1(/ml)
0
1 pg
70 pg
1 ng
35 ng
35 ng/ml TGF-β1
0
6
12
24 (hours)
250KDa
150KDa
100KDa
β-actin


F
Control
Injury
370 bp
240 bp
400 bp
500 bp
TGF-β1
bFGF
EGF
GAPDH

productions may contribute in part to the increase in KS- and CS-reactivity on Western blot.

We then examined the expression of the cytokines listed in Fig. 4A *in vivo* after brain injury. TGF-β_1 expression was found to be induced *in vivo* (Fig. 4F). bFGF and EGF are well-known cytokines that activate astrocytes (Faber-Elman et al., 1996; Smith and Strunz, 2005). bFGF, but not EGF, showed a striking increase of expression in our injury model (Fig. 4F). The induction of TGF-β_1 and bFGF after brain injury is consistent with previous reports (Wiessner et al., 1993; Endoh et al., 1994). Taking into account that TGF-β_1 expression, but not EGF expression, was induced after brain injury although both molecules induced KS and CS biosynthesis in microglias (Figs. 4A–F), our data suggested that TGF-β_1 was an intrinsic factor that could upregulate KS and CS biosynthesis *in vivo*.

2.4. *Cross talk between astrocytes and microglias*

TGF-β_1 might not only upregulate KS and CS biosynthesis, but also play a role in intercellular communication. In this context, it is noteworthy that expression of TGF-β_1 and bFGF was increased after brain injury (Fig. 4F). bFGF activates astrocytes (Meiners et al., 1993; Faber-Elman et al., 1996; Riboni et al., 2001; Brambilla et al., 2003), and primary cultured microglias can express bFGF (Araujo and Cotman, 1992). Based on these backgrounds, we considered a possible link between TGF-β_1 and bFGF. Thus, we first examined whether or not bFGF expression was induced in microglias by TGF-β_1. TGF-β_1 exposure for 24 h induced bFGF expression in primary cultured microglias in a dose-dependent manner (Fig. 5). We next addressed a complementary question: whether or not bFGF-activated astrocytes produced TGF-β_1. Astrocytes were primary cultured either with or without bFGF for 24 h, and the conditioned medium was collected (Fig. 6A). We detected the apparent induction of TGF-β_1 production by astrocytes after stimulation with bFGF (Fig. 6B). The conditioned medium was then applied to primary cultured microglias. Western blot analysis confirmed that medium conditioned from bFGF-activated astrocytes (bFGF-ACM), but not astrocytes without bFGF treatment (ACM), induced the expression of proteoglycans bearing KS and CS (Figs. 6C, D). Although bFGF alone also induced KS and CS, the extent of induction was much lower than that by bFGF-ACM. KS and CS expression induction was confirmed by immunocytochemistry (Fig. 6E). The antibody that neutralizes TGF-β_1 functions effectively blocked these inductions (Fig. 6F), indicating that the conditioned medium from bFGF-activated astrocytes indeed contained functional TGF-β_1.

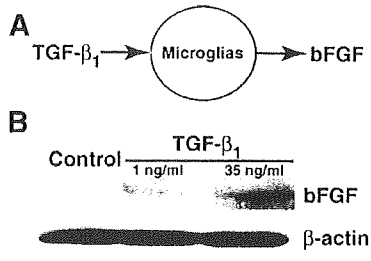


Fig. 5 – bFGF expression in primary cultured microglias by TGF-b_1. (A and B) Medium was collected from primary cultured microglias 24 h after TGF-β_1 stimulation, and subjected to Western blot analysis for bFGF expression. To make it sure that the samples were from cells with similar numbers, cell lysates were examined for β-actin expression on Western blot analysis. bFGF expression in primary cultured microglias was induced by exogenous TGF-β_1 in a dose-dependent manner. Experiments were performed 3 times and similar results were obtained.

3. Discussion

In this study, we identified microglias as a suitable target by which to investigate KS and CS expression. We found that TGF-β_1 induces both KS and CS biosyntheses in microglias. Thus, this is the first report showing that the biosyntheses of KS and CS induced after neuronal injury share a regulation mechanism in common, which is mediated by TGF-β_1 (Fig. 7). As aggrecan mRNA expression is also induced by TGF-β_1 (Fig. 4), the expression of core proteins of proteoglycans might utilize this mechanism. These findings suggest that a common regulatory mechanism contributes to biosynthesizing all components of KS/CSPGs (i.e., KS, CS and core proteins), which function as inhibitory molecules as to axonal regeneration after injury.

In interpreting our data, we suggest that upregulation of expression is responsible for the increase in levels of the enzymes. But we cannot rule out the possibility that the distribution of cell types is altered following injury and the levels of enzymes simply reflect this change. However, *in vitro* studies verified that primary cultured microglias barely express KSPG or CSPG without the stimulation of TGF-β_1, whereas expressions of enzymes for KS and CS biosynthesis are indeed upregulated by TGF-β_1. Immunohistochemistry data indicated that the main source of KSPG is microglias. Therefore, our study may suggest that the increased expression through TGF-β_1 may at least in part contribute to the increase in levels of the enzymes.

The extracellular matrix of the adult CNS has a unique composition. Instead of collagens, laminin-1 and fibronectin, this matrix is rich in hyaluronic acid and CSPGs (Ruoslahti, 1996). KSPGs and proteoglycans bearing both KS and CS chains are also important components of the CNS extracellular matrix. Disorganized re-induction of proteoglycan production may be triggered upon injury and thereby inhibit neuronal axon regrowth. From this point of view, it is important to note that a common mechanism works in the induction of KS and CS expression after neuronal injuries. This finding is interesting, as KS ablation and CS ablation independently promote functional recovery after spinal cord injury (Bradbury et al., 2002; Imagama et al., unpublished data). These collectively suggest a close functional relationship between KS and CS. For example, proteoglycans bearing both KS and CS chains, other than CS-alone or KS-alone proteoglycans, might play an important role in inhibiting axonal regeneration.

TGF-β_1 also induced bFGF expression in microglias. bFGF, in turn, induced TGF-β_1 expression in astrocytes. Indeed,

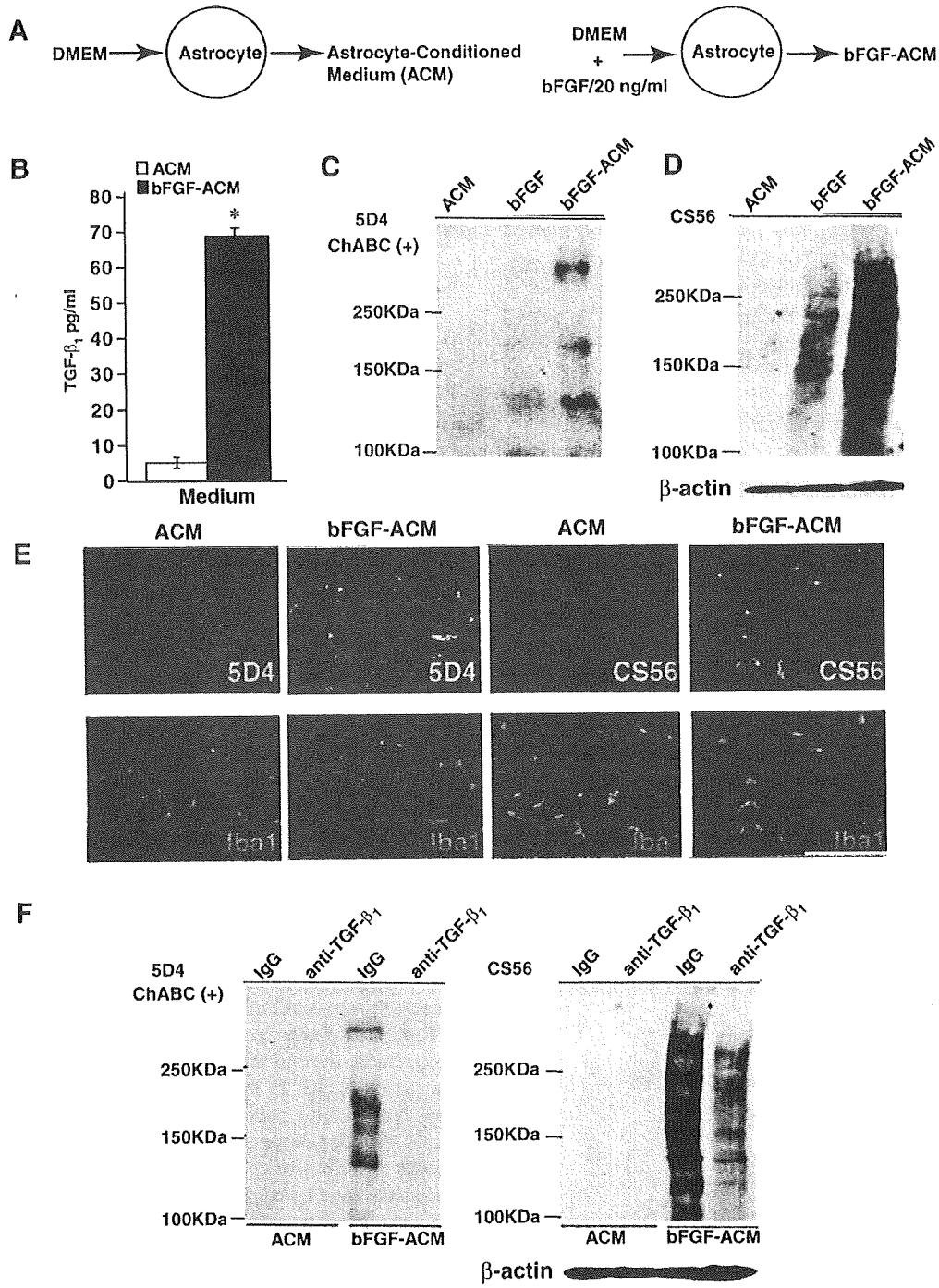


Fig. 6 – Cross talk between astrocytes and microglias through TGF-b_1 and bFGF. (A) Preparation of astrocyte-conditioned medium (ACM). Primary cultured astrocytes were incubated either with or without bFGF for 24 h. (B) TGF-β_1 expression in primary cultured astrocytes was induced by exposure to bFGF-treated ACM (bFGF-ACM). TGF-β_1 amounts were determined with ELISA specific for TGF-β_1. The results represent means±SD (n=3). *P<N0.05 (versus ACM). (C, D) KSPG and CSPG expression was induced in primary cultured microglias incubated with bFGF-ACM. Western blotting data are shown. β-actin controls are common for the 5D4 and CS56 Western blots, since the sources of the samples are the same. (E) Immunocytochemical staining for KS and CS confirmed the induction of KSPG and CSPG expression in primary cultured microglias incubated with bFGF-ACM. Bar, 150 μm. (F) TGF-β_1-neutralizing antibody diminished KS and CS expression in microglias treated with bFGF-ACM.