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ORIGINAL ARTICLE

Nogo-A and Nogo Receptor Expression in Demyelinating Lesions of Multiple Sclerosis

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Abstract

A myelin-associated neurite outgrowth inhibitor, Nogo-A, plays a key role in inhibition of axonal regeneration following injury and ischemia in the central nervous system (CNS). Because axonal injury is a pathologic hallmark of multiple sclerosis (MS), we have investigated the expression of Nogo-A and its receptor NgR in four MS and 12 non-MS control brains by immunohistochemistry. Nogo-A expression was markedly upregulated in surviving oligodendrocytes at the edge of chronic active demyelinating lesions of MS and ischemic lesions of acute and old cerebral infarction, whereas NgR expression was greatly enhanced in reactive astrocytes and microglia/macrophages in these lesions when compared with their expression in the brains of neurologically normal controls. Nogo-A and NgR were also identified in a subpopulation of neurons. In contrast, Nogo-A was undetectable in reactive astrocytes and microglia/macrophages and NgR was not expressed on oligodendrocytes in any cases examined. Western blot analysis and double labeling immunocytochemistry identified the constitutive expression of NgR in cultured human astrocytes. These results suggest that Nogo-A expressed on oligodendrocytes might interact with NgR presented by reactive astrocytes and microglia/macrophages in active demyelinating lesions of MS, although biologic effects caused by Nogo-A/NgR interaction among glial cells remain unknown.

Key Words: Axonal regeneration, Multiple sclerosis, Nogo-A, Nogo receptor, Oligodendrocytes, Reactive astrocytes

INTRODUCTION

The adult mammalian central nervous system (CNS) has an extremely limited capacity to regenerate axons following injury. The reduced regenerative ability is attributable to the progressive disappearance of growth-promoting factors or the increasing appearance of growth-inhibitory molecules during maturation of the CNS (1). Recently, Nogo is identified as a myelin-associated inhibitor for axonal regeneration (2, 3). The Nogo gene encodes three distinct isoforms, named Nogo-A, -B, and -C, derived by alternative splicing and promoter usage. All of these share a small segment composed of 66 amino acid residues located between the two putative transmembrane domains named Nogo-66, in the C-terminal region homologous to the members of reticulon protein family (2, 3). Nogo-A, the largest isoform, is predominantly expressed on oligodendrocytes and their processes with location in the innermost adaxonal and outermost myelin membranes (4, 5). Nogo-A is also identified in a subpopulation of neurons with the subcellular location chiefly in the endoplasmic reticulum (ER) and the Golgi complex, concentrated at the postsynaptic density (6–9). Nogo-B shows a ubiquitous distribution pattern, while Nogo-C, the shortest isoform, is enriched in skeletal muscle (4, 10). Nogo-A has at least two discrete regions with neuronal growth-inhibitory activities: one is located in the Nogo-A-specific region spanning amino acids 544–725 that restricts neurite outgrowth; the other, Nogo-66, has the capacity to induce growth cone collapse (11). Both regions assume different membrane topologies depending on cell types (11). Nogo-66 binds to a high affinity receptor NgR, a glycoprotein composed of a signal sequence, a leucine-rich repeat (LRR)-type N-terminal region (LRRNT), eight LRR domains, a cysteine-rich LRR-type C-terminal domain (LRRCT), a unique C-terminal domain, and a glycosylphosphatidylinositol (GPI) anchorage site responsible for accumulation in lipid rafts (12, 13). NgR expression is sufficient to confer sensitivity to Nogo-66 on otherwise insensitive cells (12). In contrast to Nogo-A, NgR is not identified on oligodendrocytes but is expressed constitutively in a subset of neurons and their axons, including cerebral cortical pyramidal neurons and cerebellar Purkinje cells (12, 14, 15). Signal transduction mediated by NgR depends on its association with the low-affinity nerve growth factor receptor $p75^{NTR}$, which also serves as a coreceptor for the Trk family of neurotrophin receptors. Recent studies showed that not only Nogo-66 but also myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp) bind to NgR and transduce neurite growth-inhibitory signals via $p75^{NTR}$ by activating RhoA and inhibiting Rac1 (16, 17). By neutralizing anti-Nogo-A antibodies, NgR competitive antagonistic peptides, or soluble truncated NgR, in vivo blockade of the interaction between NgR and its

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ligands induced long-distance axonal regeneration, compensatory sprouting, and upregulation of growth-associated genes (18, 20). This was accompanied by enhancement of functional recovery after injury in the CNS (18–20).

Multiple sclerosis (MS) is pathologically characterized by multifocal inflammatory demyelination and axonal injury in the CNS white matter; the latter has been proposed as a principal cause of permanent disability in MS (21, 22). A recent study identified anti-Nogo-A autoantibody in the serum and cerebrospinal fluid of relapsing-remitting MS patients, suggesting a protective response to persistent demyelination and axonal damage (23). However, it remains unknown whether Nogo-A, MAG, and OMgp play an active role in interfering with axonal regeneration at the site of demyelinating lesions of MS. Previous studies suggested that Nogo-A in the intact adult rodent CNS regulates axonal plasticity and stabilizes major myelinated tracts to prevent the formation of aberrant fiber connections (24, 25). To investigate a physiological function of Nogo-A in development and maturation of the CNS, three independent lines of Nogo-A knockout mice have been established recently (26–28). Unexpectedly, all of these mice showed neither obvious neuroanatomic defects nor neurologic symptoms, indicating that Nogo-A is not pivotal for development and maintenance of axonal pathways at least in the absence of injury. Following spinal cord injury, some lines of Nogo-A-deficient mice showed an enhanced axonal regeneration of corticospinal tract fibers (26, 27). Importantly, inflammatory demyelination and axonal damage were less severe in Nogo-A-deficient mice affected with experimental autoimmune encephalomyelitis, an animal model of MS (29). Furthermore, NgR-deficient mice exhibited an enhanced axonal plasticity after ischemic stroke in the brain, accompanied by improved functional recovery (30). These observations suggest that Nogo-A and NgR interaction plays a central role in inhibition of axonal regeneration under pathologic conditions in the CNS.

In the present study, we have investigated the expression of Nogo-A and NgR in MS brains by immunohistochemistry. We found that Nogo-A expression was markedly upregulated in surviving oligodendrocytes at the edge of chronic active demyelinating lesions of MS, while NgR expression was greatly enhanced in reactive astrocytes and microglia/macrophages in these lesions. Our observations suggest a novel type of interaction between Nogo-A on oligodendrocytes and NgR on activated astrocytes and microglia at the site of demyelinating lesions of MS.

MATERIALS AND METHODS

MS and Control Brain Tissues

Ten-micron-thick serial sections were prepared from autopsied brains of 4 MS cases, 6 non-MS neurologic and psychiatric disease (OND) cases, and 6 neurologically normal control subjects listed in Table 1. Detailed clinical and neuroradiologic profiles of MS patients were described previously (31). The tissues were fixed with 4% paraformaldehyde or 10% neutral formalin and embedded in paraffin. Autopsies on all subjects were performed at the National Center Hospital for Mental, Nervous and Muscular Disorders, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan. Written informed consent was obtained in all cases. The present study was approved by the Ethics Committee of NCNP.

Immunohistochemistry and Immunocytochemistry

After deparaffinization, tissue sections were heated by microwave at 95°C for 10 minutes in 10 mmol/L citrate sodium buffer, pH 6.0. They were then treated at room temperature (RT) for 15 minutes with 3% H_2O_2-containing methanol. For $p75^{NTR}$ immunolabeling, the tissue sections

TABLE 1. MS and Control Cases Examined in the Present Study

Case No.	Age (year) and Sex (male/female)	Diagnosis	Cause of Death
791	29 F	Secondary progressive multiple sclerosis	Asphyxia
744	40 F	Secondary progressive multiple sclerosis	Respiratory failure
609	43 F	Primary progressive multiple sclerosis	Hyperglycemia
544	33 M	Secondary progressive multiple sclerosis	Sepsis and multiorgan failure
719	47 M	Acute cerebral infarction	Sepsis
786	84 M	Acute cerebral infarction	Disseminated intravascular coagulation
789	62 M	Old cerebral infarction	Pancreatic cancer
807	56 M	Old cerebral infarction	Myocardial infarction
523	36 F	Schizophrenia	Lung tuberculosis
826	61 M	Schizophrenia	Asphyxia
G6	79 F	Neurologically normal subject	Hepatic cancer
G7	75 F	Neurologically normal subject	Breast cancer
G8	60 F	Neurologically normal subject	External auditory canal cancer
G9	74 F	Neurologically normal subject	Gastric and hepatic cancers
A2623	83 F	Neurologically normal subject	Gastric cancer and myocardial infarction
A2647	65 M	Neurologically normal subject	Liver cirrhosis and bronchopneumonia

The present study includes four MS cases numbered 791, 744, 609, and 544, 6 non-MS neurologic and psychiatric disease cases (OND) numbered 719, 786, 789, 807, 523, and 826, and 6 neurologically normal cases (NNC) numbered G6, G7, G8, G9, A2623, and A2647.

were pretreated with 0.125% trypsin solution (Nichirei, Tokyo, Japan) at 37°C for 10 minutes. They were incubated with 10% normal goat serum-containing phosphate-buffered saline (PBS) at RT for 15 minutes to block nonspecific staining. The sections were incubated in a moist chamber at 4°C overnight with primary antibodies listed in Table 2. After washing with PBS, they were labeled at RT for 30 minutes with peroxidase-conjugated secondary antibodies (Nichirei) followed by incubation with a colorizing solution containing diaminobenzidine tetrahydrochloride and a counterstain with hematoxylin. For negative controls, tissue sections were incubated with a rabbit negative control reagent (DAKO, Carpinteria, CA) instead of primary antibodies.

For immunocytochemistry, human astrocytes in culture on cover glasses were fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4 at RT for 10 minutes, followed by incubation with PBS containing 0.5% Triton X-100 at RT for 20 minutes. For double immunolabeling, the cells and tissue sections were incubated at RT for 30 minutes with a mixture of rabbit anti-NgR antibody and mouse anti-GFAP antibody. Then, they were incubated at RT for 30 minutes with a mixture of rhodamine-conjugated anti-rabbit IgG and FITC-conjugated mouse IgG (ICN-Cappel, Aurora, OH). After several washes, they were mounted with glycerol-polyvinyl alcohol and examined under a Nikon ECLIPSE E800 universal microscope equipped with fluorescein and rhodamine optics. Negative controls were processed following all the steps except for exposure to primary antibody. In some experiments, tissue sections were initially stained with rabbit anti-Nogo-A antibody, then followed by incubation with alkaline phosphatase-conjugated secondary antibody (Nichirei) and colorized with New Fuchsin substrate. After inactivation of all the antibodies by heating the sections at 95°C for 10 minutes in 10 mmol/L citrate sodium buffer, pH 6.0, they were relabeled with rabbit anti-MBP antibody, followed by incubation with peroxidase-conjugated secondary antibody (Nichirei) and colorized with diaminobenzidine tetrahydrochloride substrate.

Cell Culture and Expression of Transgenes

Cultured human astrocytes derived from human neuronal progenitor cells were maintained in Dulbecco's Modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (feeding medium), as described previously (31). To enrich the GPI-anchored proteins, astrocytes were incubated at 37°C for 3 hours in the serum-free Dulbecco's Modified Eagle's medium /F-12 medium (Invitrogen) supplemented with 5 U/mL phosphatidylinositol-specific

TABLE 2. Primary Antibodies Used for Immunochemistry and Western Blot Analysis

Antibody (clone name)	Supplier	Code	Origin	Immunogens	Antigen Specificity	Concentration Used for Immunohistochemistry	Concentration Used for Western Blotting
Nogo-A	Santa Cruz Biotechnology	sc-25600	Rabbit	Peptide composed of amino acids 700–1,000 mapping at the internal region of human Nogo-A	Nogo-A not reactive with Nogo-B or Nogo-C	1:2,000 (100 ng/mL)	1:12,000 (16.7 ng/mL)
NgR	Chemicon	AB5615	Rabbit	Recombinant mouse NgR	NgR	1:2,000	1:4,000
$p75^{NTR}$ (ME20.4)	Sigma	N5408	Mouse	Human melanoma cell line	Low affinity nerve growth factor receptor p75	1:500 (46 μg/mL)	NA
APP (22C11)	Chemicon	MAB348	Mouse	Recombinant human APP	APP	1:200 (5 μg/mL)	NA
GFAP	Dako	N1506	Rabbit	Purified bovine spinal cord GFAP	GFAP	Prediluted	NA
GFAP (GA5)	Nichirei	422261	Mouse	Purified swine spinal cord GFAP	GFAP	Prediluted	NA
MBP	Dako	N1564	Rabbit	Purified human brain MBP	MBP	Prediluted	NA
NF (2F11)	Nichirei	412551	Mouse	Purified human brain NF protein	Human 70-kDa and 200-kDa NF	Prediluted	NA
CD68 (KP1)	Dako	N1577	Mouse	Lysosomal granules of human lung macrophages	CD68	Prediluted	NA
CD3 (PS1)	Nichirei	413241	Mouse	Recombinant human CD3 epsilon chain	CD3	Prediluted	NA
HSP60	Santa Cruz Biotechnology	sc-1052	Goat	Peptide mapping at the amino terminus of human HSP60	HSP60	NA	1:2,000 (100 ng/mL)

NgR, Nogo receptor; NTR, neurotrophin receptor; APP, amyloid precursor protein; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; NF, neurofilament; HSP60, 60-kDa heat shock protein; NA, not applied.

phospholipase C (PI-PLC; Sigma, St. Louis, MO). The culture supernatant was harvested and concentrated at a 1/30 volume by centrifugation on a Centricon-10 filter (Millipore, Bedford, MA). Human cell lines such as HEK293 embryonal kidney cells, U-373MG astrocytoma, and NTera2 teratocarcinoma were obtained from the RIKEN Cell Bank (Tsukuba, Japan) and the American Type Culture Collection (Rockville, MD). In limited experiments, human astrocytes and U-373MG cells were incubated for 4 to 8 days in the serum-free Dulbecco's Modified Eagle's medium /F-12 medium supplemented with an insulin-transferrin-selenium supplement (Invitrogen) with or without inclusion of recombinant human IL-1β or TNFα (PeproTech EC, London, UK).

For expression of transgenes, the human *Nogo-A* gene (GenBank accession no. NM_020532) encoding the Nogo-A-specific segment (NAS; amino acids 186–1004) and the human *NgR* gene (NM_023004) encoding the full-length NgR after a cleavage of the N-terminal signal peptide (amino acids 27–473) were amplified by PCR using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) from cDNA of NTera2-derived human neurons (32) using sense and antisense primer sets (5′gatgagacccttttgctcttcct3′ and 5′tcatgaagttttactcagctctgctga3′ for Nogo-A and 5′acgatggagagggcgtccgctggag3′ and 5′gcagggcccaagcactgtccacagcac3′ for NgR). The *Nogo-A* or *NgR* gene was cloned in an expression vector pcDNA4/HisMax-TOPO containing a N-terminal Xpress tag for detection of the recombinant protein or in pEF6/V5-His-TOPO containing a C-terminal V5 tag (Invitrogen), respectively. The vectors were transfected into HEK293 cells by using Lipofectamine 2000 reagent (Invitrogen). At 48 hours after transfection, the cells were processed for Western blot analysis.

For RT-PCR analysis, cDNA was amplified for 30 cycles by PCR using sense and antisense primer sets specific for the human NgR gene (5′cagtacctgaggctcaacgacaac3′ and 5′acctgagccttctgagtcaccagt3′; product size 588 bp) or the human glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) gene (NM_002046; 5′ccatgttcgtcatgggtgtgaacca3′ and 5′gccagtagaggcagggatgatgttc3′; the product size 251 bp) (32).

Western Blot Analysis

To prepare total protein extract, the cells and tissues were homogenized in RIPA lysis buffer composed of 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, and a cocktail of protease inhibitors (Roche Diagnostics, Mannheim, Germany), followed by centrifugation at 12,000 rpm for 20 minutes at RT. The supernatant was collected for separation on a 6%, 8%, or 12% SDS-PAGE gel. The protein concentration was determined by a Bradford assay kit (BioRad, Hercules, CA). After gel electrophoresis, the protein was transferred onto nitrocellulose membranes and immunolabeled at RT overnight with rabbit anti-Nogo-A or anti-NgR antibody. The membranes were incubated at RT for 30 minutes with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The specific reaction was visualized with a Western blot detection system using a chemiluminescent substrate (Pierce, Rockford, IL). After the antibodies were stripped by incubating the membranes at 50°C for 30 minutes in stripping buffer composed of 62.5 mmol/L Tris-HCl, pH 6.7, 2% SDS, and 100 mmol/L 2-mercaptoethanol, the membranes were processed for relabeling with goat anti-HSP60 antibody followed by incubation with horseradish peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnology), or with mouse monoclonal anti-Xpress or anti-V5 antibody (Invitrogen) followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology).

RESULTS

Characterization of Anti-Nogo-A and Anti-NgR Antibodies

To characterize the specificity of polyclonal anti-Nogo-A antibody (sc-25600) and anti-NgR antibody (AB5615) (Table 2), we investigated Nogo-A and NgR expression in brain homogenates by Western blot analysis. The antibody sc-25600 was raised against a peptide consisting of amino acids 700–1,000 of the human Nogo-A that represents a Nogo-A-specific internal segment not shared with Nogo-B or Nogo-C. This antibody reacted with a single band of 190-kDa protein in human and mouse brain and spinal cord homogenates (Fig. 1A). This size corresponds to that of the full-length Nogo-A of the rat oligodendrocyte lysate (11). The antibody sc-25600 recognized a 140-kDa recombinant NAS protein with an Xpress tag in the vector-transfected HEK293 cells but did not react with any proteins in nontransfected HEK293 cells (Fig. 1C). The antibody AB5615 was raised against a recombinant mouse NgR. This antibody reacted with a 64-kDa protein in human and mouse brain and spinal cord homogenates (Fig. 1B). This size corresponds to that of the full-length NgR identified in the NgR gene-transfected CHO-K1 cells and SH-SY5Y cells (33, 34). The antibody AB5615 recognized not only a 64-kDa endogenous NgR protein constitutively expressed in nontransfected HEK293 cells but also reacted with several bands immunoreactive for a V5 tag in the vector-transfected HEK293 cells (Fig. 1D). The latter might represent posttranscriptionally modified NgR isoforms. In agreement with detection of NgR in HEK293 cells on immunoblot, RT-PCR analysis using NgR-specific primer sets, which do not amplify NgR homologues NgRH1 and NgRH2 (33), identified the constitutive expression of NgR mRNA in HEK293 cells (data not shown).

Nogo-A Expression on Oligodendrocytes in Demyelinating Lesions of MS

To investigate Nogo-A expression in MS lesions, the brain, spinal cord, and optic nerve sections of 4 progressive MS patients and 12 non-MS control cases (Table 1) were processed for immunohistochemistry using the antibody sc-25600. Adjacent sections were stained with the antibodies against cell type-specific markers. In all MS cases, a substantial population (20%–60%) of surviving oligodendrocytes and remaining myelin sheath at the edge of chronic active demyelinating lesions, where numerous $CD68^+$ macrophages/microglia accumulated (Fig. 2a inset), expressed an intense immunoreactivity for Nogo-A (Table 3; Figure 2a and 2b inset). In contrast, a smaller population (< 20%) of oligodendrocytes

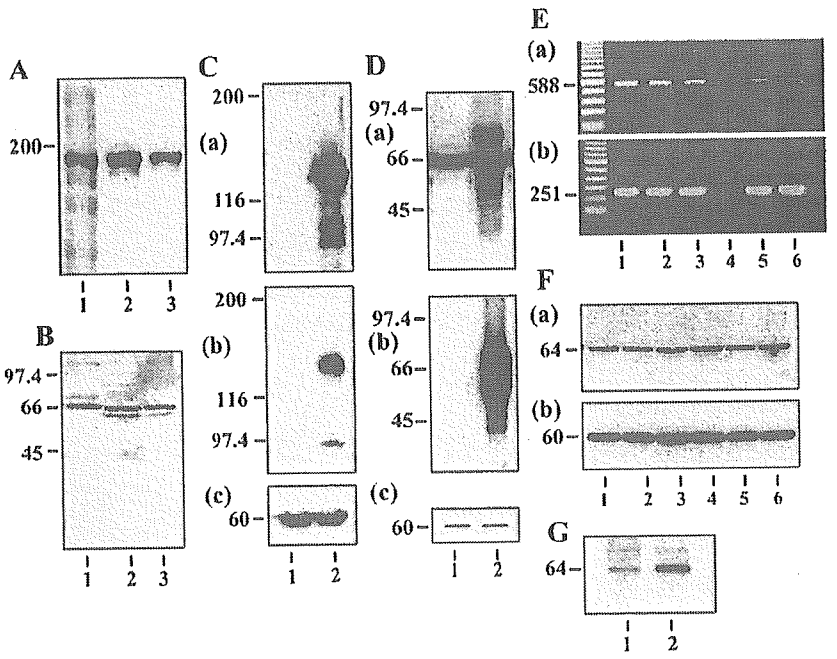

FIGURE 1. Nogo-A and NgR expression in human brain, cultured astrocytes, transfected HEK293 cells. **(A, B)** Immunoblot of brain homogenates with anti-Nogo-A **(A)** or anti-NgR **(B)** antibody. Human (lane 1) or mouse brain (lane 2), and mouse spinal cord (lane 3), 20 μg of protein each. **(C, D)** Immunoblot of HEK293 cells expressing Nogo-A-specific segment (NAS) **(C)** or NgR **(D)** with **(Ca)** anti-Nogo-A, **(Cb)** anti-Xpress, **(Da)** anti-NgR, **(Db)** anti-V5, or **(Cc** and **Dc)** anti-HSP60 antibody. Nontransfected (lane 1) and transfected (lane 2) HEK293 cells, 120 μg **(C)** or 4 μg **(D)** of protein each. **(E)** RT-PCR analysis of **(Ea)** NgR and **(Eb)** G3PDH mRNA in human astrocytes, astrocytoma, and teratocarcinoma cell lines. The cells were incubated for 8 days in serum-free (lanes 2, 5) or 10% FBS-containing (lanes 1, 3, 4, 6) medium. NTera2 (lane 1), cultured human astrocytes (lanes 2–4), and U-373MG (lanes 5, 6) with (lanes 1–3, 5, 6) or without (lane 4) inclusion of RT step. **(F)** Immunoblot of cultured human astrocytes with **(Fa)** anti-NgR or **(Fb)** anti-HSP60 antibody. The cells were incubated for 4 days in serum-free (lanes 1–3) or 10% FBS-containing (lanes 4–6) medium with inclusion of 100 ng/mL IL-1β (lanes 2, 5) 100 ng/mL TNF-α (lanes 3, 6) or without cytokines (lanes 1, 4), 30 μg of protein each. **(G)** Immunoblot of supernatant of cultured human astrocytes with anti-NgR antibody. The supernatant of (lane 1) untreated and (lane 2) PI-PLC-treated astrocytes.

distributed in the white matter of the brains of neurologically normal subjects displayed a fairly weak immunoreactivity for Nogo-A (Fig. 2e). An intense Nogo-A immunoreactivity was also found in surviving oligodendrocytes at the lesion border of acute and old cerebral infarction (Fig. 2d inset). The number of Nogo-A-expressing oligodendrocytes was much smaller in the center of demyelinating lesions and in the normal-appearing white matter of MS brains (Fig. 2d) and in the necrotic lesions of cerebral infarction. Double labeling immunohistochemistry verified a close association between Nogo-A-expressing oligodendrocytes and MBP, an interacting partner of Nogo-A (35) in MS lesions (Fig. 2b inset). In all MS and non-MS cases, variable Nogo-A immunoreactivity was identified in a small population (< 20%) of neurons widely distributed in the whole CNS, including motor neurons in the spinal cord with its location in the perikarya and neurites (Table 3; Fig. 2f), suggesting that not all but a substantial population of neurons in the adult human CNS express Nogo-A constitutively. In contrast, Nogo-A expression was undetectable in $GFAP^+$ reactive astrocytes (Fig. 2a, c), $CD68^+$ microglia/macrophages, ependymal cells, or $CD3^+$ T lymphocytes in chronic active demyelinating lesions of MS, ischemic lesions of cerebral infarction, and other cases (Table 3).

To investigate a possible association of Nogo-A-expressing oligodendrocytes with damaged axons in active MS lesions, adjacent sections were stained with the antibody against amyloid precursor protein (APP), a sensitive marker for acute axonal injury (21). However, APP-immunoreactive axons were hardly detectable in any cases examined (Fig. 3b), and these axons did not colocalize with Nogo-A-expressing oligodendrocytes (not shown).

NgR Expression on Reactive Astrocytes and Microglia in Demyelinating Lesions of MS

To investigate NgR expression in MS lesions, the brain, spinal cord, and optic nerve sections of 4 MS patients and 12 non-MS control cases (Table 1) were processed for immunohistochemistry using the antibody AB5615. In all MS and cerebral infarction cases, a large population (> 60%) of $GFAP^+$ reactive astrocytes and $CD68^+$ microglia/macrophages that accumulated in chronic active and inactive demyelinating lesions or in ischemic lesions expressed an intense immunoreactivity for NgR (Table 3; Fig. 4a, d, e). Furthermore, a fairly small number of $GFAP^+$ astrocytes and $CD68^+$ microglia, occasionally found in the brains of neurologically normal subjects, were also stained intensely with anti-NgR antibody (Fig. 4d inset). These observations suggest that both astrocytes and microglia express high levels of NgR, particularly when they become activated. Double-labeling immunohistochemistry verified coexpression of NgR and GFAP on reactive astrocytes in demyelinating lesions of MS (Fig. 3c, d). In all MS and non-MS cases, variable NgR immunoreactivity was identified in a large population (> 60%) of neurons and their neurites widely distributed in the whole CNS (Table 3; Fig. 4f), suggesting that not all but a wide variety of neurons in the adult human CNS constitutively express high levels of NgR. Among them, cerebral cortical neurons and spinal cord motor neurons coexpressed Nogo-A and NgR (Figs. 2f, 4f). In addition,

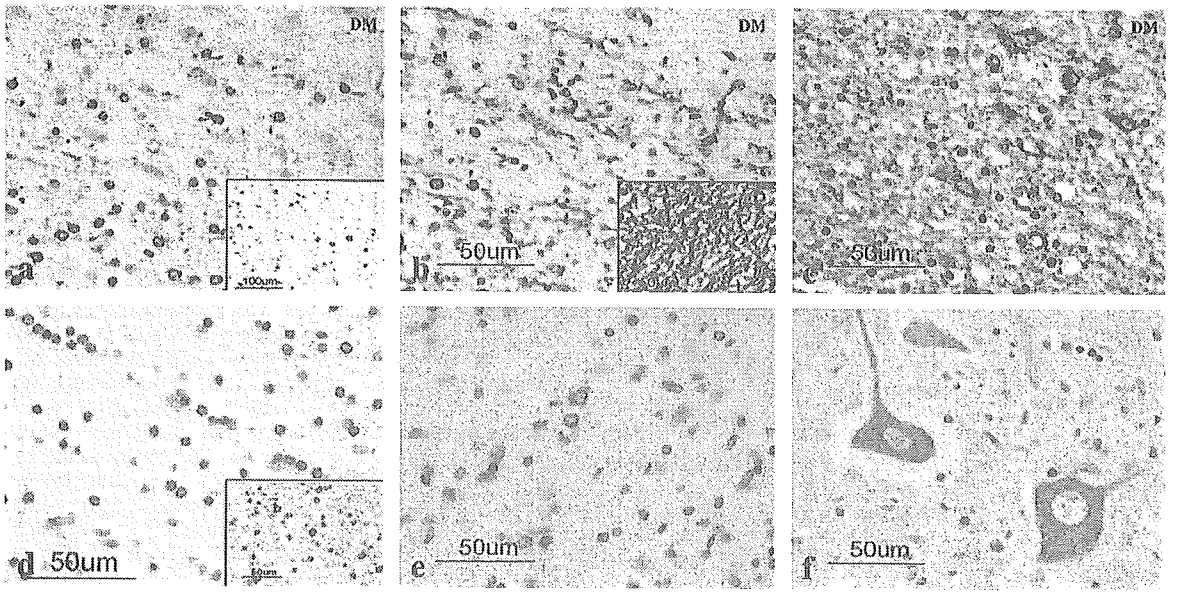

FIGURE 2. Nogo-A expression on oligodendrocytes in demyelinating lesions of MS. Immunohistochemistry. **(a)** No. 744 MS, Nogo-A, the edge of chronic active demyelinating lesions (DM) in the subcortical white matter of the frontal lobe (inset, CD68). **(b)** No. 744 MS, MBP, the adjacent section of panel a (inset, no. 791 MS, double immunolabeling for Nogo-A as red and MBP as brown). **(c)** No. 744 MS, GFAP, the adjacent section of panel a. **(d)** No. 744 MS, Nogo-A, the normal-appearing white matter of the frontal lobe (inset, no. 786 acute cerebral infarction, Nogo-A, the lesion border in the subcortical white matter of the parietal lobe). **(e)** No. A2647 neurologically normal subject, Nogo-A, the subcortical white matter of the frontal lobe. **(f)** No. 791 MS, Nogo-A, motor neurons in the spinal cord.

ependymal cells constitutively expressed intense NgR immunoreactivity, while NgR expression was not found in oligodendrocytes (Table 3; Fig. 4a, c) or $CD3^+$ T lymphocytes.

In contrast to widespread distribution of NgR in the human CNS, the NgR coreceptor $p75^{NTR}$ immunoreactivity was identified in fairly restricted regions: most prominently expressed in nerve fibers of substantia gelatinosa in the spinal cord (Fig. 3a), tractus solitarius in the brainstem, and found in the vascular wall in the cerebrum. $p75^{NTR}$ was not expressed on oligodendrocytes, astrocytes, or microglia/macrophages in any cases examined (not shown).

Constitutive Expression of NgR in Cultured Human Astrocytes

Because a previous study did not identify NgR on astrocytes in the human CNS (15), NgR expression was studied in cultured human astrocytes to verify the present observations. RT-PCR analysis using NgR-specific primer sets identified a substantial level of NgR mRNA in human astrocytes in culture, along with U-373MG and NTera2 cells (Fig. 1E). No products were amplified when total RNA was processed for PCR omitting RT step, confirming that a contamination of genomic DNA was excluded (Fig. 1E, lane 4). By Western blot analysis, NgR protein levels were unaltered in cultured human astrocytes by exposure to IL-1β or TNFα under the serum-free or serum-containing culture condition, when standardized against the levels of HSP60, a housekeeping gene product, detected on the identical blots (Fig. 1F). Double labeling immunocytochemistry verified coexpression of NgR and GFAP in cultured human astrocytes, where a substantial NgR immunoreactivity was identified in the cytoplasm (Fig. 3e, f). Furthermore, a large amount of NgR protein was detected in the supernatant of PI-PLC-treated

TABLE 3. Differential Expression of Nogo-A and Nogo Receptor in Glial Cells and Neurons in MS and Control Brains

Brain	Astrocytes			Microglia/Macrophages			Oligodendrocytes			Neurons		
	MS	OND	NNC	MS	OND	NNC	MS	OND	NNC	MS	OND	NNC
Nogo-A	n(−)	n(−)	n(−)	n(−)	n(−)	n(−)	m(++)	m(++)	s(+)	s(+/++)	s(+)	s(+)
NgR	l(++)	l(++)	l*(++)	l(++)	l(++)	l*(++)	n(−)	n(−)	n(−)	l(+/++)	l(+/++)	l(+/++)

The present study includes 4 MS cases, 6 non-MS neurologic and psychiatric disease cases (OND), and 6 neurologically normal cases (NNC), as shown in Table 1. The population size of immunoreactive cells per total is expressed as [l] large (>60%); [m] moderate (60–20%); [s] small (<20%), [n] almost none, and [l*] large population but small number. The intensity of immunoreactivity is graded as (−) negative, (+) weak, (++) intense, and (+/++) variable.

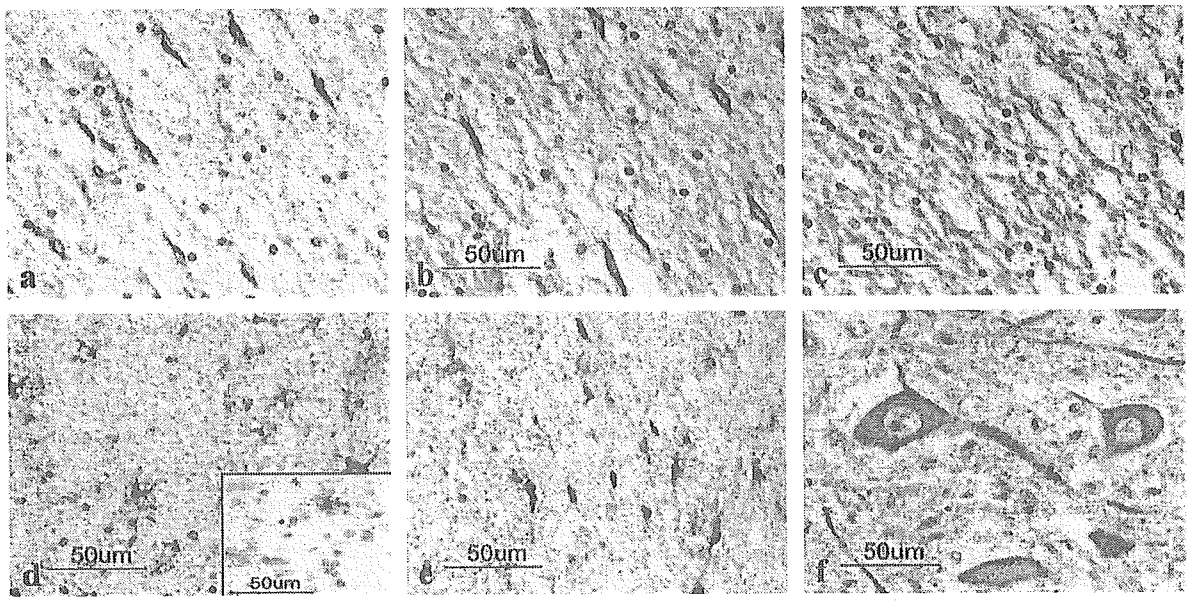

FIGURE 3. NgR expression on reactive astrocytes and microglia in demyelinating lesions of MS. Immunohistochemistry. **(a)** No. 744 MS, NgR, the edge of chronic active demyelinating lesions in the subcortical white matter of the frontal lobe. **(b)** No. 744 MS, GFAP, the adjacent section of panel a. **(c)** No. 744 MS, MBP, the adjacent section of panel a. **(d)** No. 791 MS, NgR, chronic active demyelinating lesions in the pons (inset, no. A2647 neurologically normal subject, NgR, the subcortical white matter of the frontal lobe). **(e)** No. 744 MS, NgR, chronic active demyelinating lesions in the pons. **(f)** No. 744 MS, NgR, motor neurons in the spinal cord.

human astrocyte cultures (Fig. 1G, lane 2), although a small amount of NgR was found in that of untreated cultures (Fig. 1G, lane 1).

DISCUSSION

The present study showed that Nogo-A expression was markedly upregulated in surviving oligodendrocytes, while NgR expression was greatly enhanced in reactive astrocytes and microglia/macrophages in chronic active demyelinating lesions of MS and ischemic lesions of acute and old cerebral infarction, when compared with their expression in the brains of neurologically normal controls. Both Nogo-A and NgR were also identified in a subpopulation of neurons in the brain and spinal cord, consistent with previous observations (4–9, 14, 15). In contrast, Nogo-A was undetectable in reactive astrocytes and microglia/macrophages, and NgR was not virtually expressed on oligodendrocytes. Previous studies suggested that Nogo-A released from injured oligodendrocytes and damaged myelin sheath in the CNS lesions acts on neighboring NgR-expressing neurons and their axons (36). Our observations raise an alternative possibility that Nogo-A expressed on surviving oligodendrocytes interacts directly with NgR presented by reactive astrocytes and microglia/macrophages at the site of active demyelinating lesions of MS. A possible role of NgR on reactive astrocytes and microglia includes an inhibition of their proliferation, down-regulation of cytokine production, and sequestration of Nogo-A released from damaged oligodendrocytes by acting as a non-functioning decoy receptor.

The regulatory mechanism for Nogo-A and NgR expression remains largely unknown. Several studies suggested that Nogo-A and NgR levels are not substantially altered in the adult rodent CNS following injury (4, 6, 14). The CNS injury is often accompanied by a local infiltration of lymphocytes and macrophages and an activation of microglia and astrocytes, all of which provide a source of reactive oxygen species, pro-inflammatory cytokines, and neurotrophic factors. Previously, we found that Nogo-A and NgR mRNA levels are unaffected in human neurons in culture by exposure to basic FGF, BDNF, GDNF, IL-1β, or TNF-α, despite their expression of specific receptors (32). The present study revealed that human astrocytes in culture constitutively express NgR, whose levels remain unchanged by treatment with IL-1β or TNFα. Recent studies showed that the expression of Nogo-A but not of NgR is regulated by stress-inducing stimuli. Global ischemia enhances Nogo-A expression on the myelin sheath in the adult rat brain (37), supporting the present observations. In contrast, neonatal hypoxia reduces Nogo-A protein levels on oligodendrocytes in a mouse model (38). Nogo-A expression is markedly reduced at CNS paranodes in the rats affected with experimental autoimmune encephalomyelitis (39). Nogo-A expression is upregulated around the lesion site, whereas NgR is maintained at constant levels in the adult mouse and rat spinal cord following injury (5, 8). Nogo-A mRNA levels are elevated in the adult rat hippocampal neurons after kainate-induced seizure (40). Furthermore, Nogo-A is induced in hippocampal neurons of the patients with temporal lobe epilepsy (41). Nogo-A is upregulated in denervated and innervated mouse skeletal muscle and in postmortem and biopsied muscles of amyotrophic lateral sclerosis patients (42, 43).

The human Nogo-A/B promoter lacks a typical TATA-box and consensus sequences for known oligodendrocyte-specific

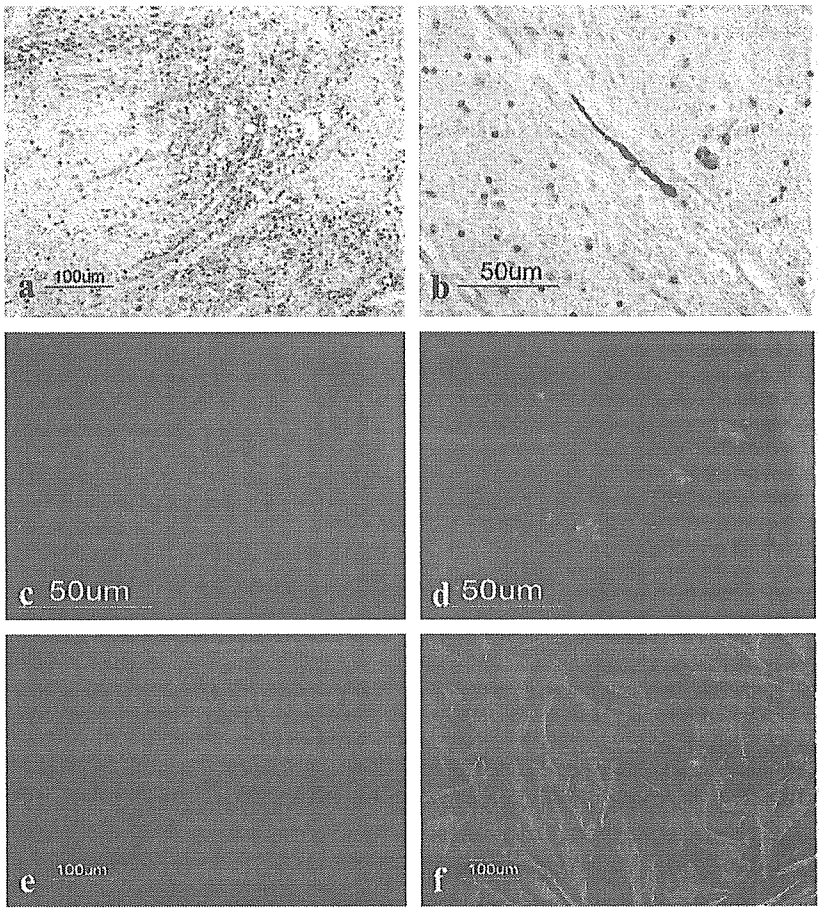

FIGURE 4. Coexpression of NgR and GFAP on reactive astrocytes in MS lesions and cultured human astrocytes. Immunohistochemistry and immunocytochemistry. **(a)** No. 791 MS, $p75^{NTR}$, the substantia gelatinosa in the spinal cord. **(b)** No. 609 MS, APP, chronic active demyelinating lesions in the medulla oblongata. **(c, d)** No. 744 MS, double immunolabeling for NgR (red) and GFAP (green), chronic active demyelinating lesions in the subcortical white matter of the frontal lobe. **(e, f)** Cultured human astrocytes, double immunolabeling for NgR (red) and GFAP (green).

transcription factors, but it has a CpG island where a number of CpG sequences are frequently methylated (10). Such GC-rich promoters are typically identified in housekeeping genes whose expression is ubiquitous. However, multiple GC-boxes within the promoter region might lead to a synergistic activation by the Sp1 family of oligodendrocyte-specific or neuron-specific transcription factors (10). Interestingly, several nonneural cell lines such as 3T3 fibroblasts and C2 myoblasts express Nogo-A, where no direct correlation is observed between Nogo-A mRNA and protein levels (10). On the other hand, the human NgR promoter has not at present been characterized. An in situ hybridization study showed that NgR is undetectable in the spinal cord, and not expressed in ependymal cells, but identified in a subpopulation of neurons in the neocortex, hippocampus, amygdala, and the dorsal root ganglia in the adult human CNS (15). The discrepancy between this study and our own is attributable to the differences in brain tissues examined and the methods applied for identification of NgR. Recent studies showed that a subset of CNS neurons, including motor neurons in the adult rat and mouse spinal cord, coexpress NgR and Nogo-A (4, 8, 14), supporting the present observations. In addition, both Nogo-A and NgR are identified in the human spinal cord during development when Nogo-A does not play a negative role in regeneration (44). Most importantly, NgR expression is not confined to neurons. U87MG human glioblastoma cells express a great amount of endogenous NgR, through which Nogo-66 modulates their growth and migration (45). Although the LRR, LRRCT, and LRRNT subdomains of NgR are all involved in ligand binding (46), the DNAQLR motif located in the third LRR domain is identified as the principal epitope recognized by a monoclonal anti-NgR antibody capable of blocking binding of all NgR ligands (47). We found that a substantial NgR immunoreactivity was located in the cytoplasm of cultured human astrocytes, in addition to the detection of NgR protein in the supernatant of PI-PLC-treated and untreated cultures. The intracellular localization of NgR appears unusual, but it might reflect the changes in cell–cell interactions under culture conditions (44). Supporting our observations, an immunoelectron microscopic analysis showed that NgR is located at both presynaptic and postsynaptic regions where NgR-immunoreactive products distribute diffusely among cytoplasmic elements including synaptic vesicles, mitochondria, and microtubules (5). Furthermore, in human neuroblastoma cells, NgR is constitutively cleaved in a post-ER compartment by zinc metalloproteinases, and a

resulting soluble N-terminal fragment is released into the culture medium (34).

The NgR coreceptor $p75^{NTR}$ transducing the signals from Nogo-66, MAG, and OMgp via NgR (16, 17) acts as a displacement factor that releases Rho from the Rho GDP dissociation inhibitor (Rho-GDI) (48). Neurons lacking $p75^{NTR}$ neither show RhoA activation nor exhibit neurite growth inhibition in the presence of myelin components (16). In general, $p75^{NTR}$ is expressed at high levels in neurons and glial cells during development, while its expression level declines to background levels in the adult CNS (49). However, $p75^{NTR}$ is reexpressed in the adult CNS following injury, ischemia, and inflammation (50). Previous studies by immunohistochemistry using a polyclonal anti-$p75^{NTR}$ antibody (G323A, Promega) showed that $p75^{NTR}$ is expressed on oligodendrocytes and microglia/macrophages in active MS lesions (51, 52). In contrast, the present study using the monoclonal antibody ME20.4 showed that $p75^{NTR}$ expression was limited in some regions such as substantia gelatinosa in the spinal cord, as described previously (53). A recent study showed that the antibody G323A exhibits a broad reactivity with numerous cross-reactive bands in PNS and CNS homogenates (54). Our observations support the view that NgR and $p75^{NTR}$ distribution does not always overlap in the CNS. NgR is identified in many cell types in the adult CNS that exhibit little or no $p75^{NTR}$ expression, whereas $p75^{NTR}$-expressing central cholinergic neurons in the medial septal nucleus do not express NgR (14). The depletion of $p75^{NTR}$ does not promote axonal regeneration after spinal cord injury (55), suggesting that an unidentified coreceptor for NgR might act as an alternative transducer of neurite growth-inhibitory signals (49). Another possibility exists that no alternative coreceptor is expressed in NgR-expressing cells where NgR acts as a nonsignaling receptor to take up an excessive amount of extracellularly released Nogo. Importantly, the NgR/$p75^{NTR}$ receptor complex is not required for mediating the neurite growth-inhibitory activity of the NAS domain of Nogo-A (56).

In conclusion, Nogo-A expression was upregulated in surviving oligodendrocytes, while NgR expression was enhanced in reactive astrocytes and microglia/macrophages in chronic active demyelinating lesions of MS, although the functional significance of these observations remains to be further investigated.

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Therapeutic Potential of Glycolipid Ligands for Natural Killer (NK) T Cells in the Suppression of Autoimmune Diseases

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Abstract: NKT cells emerge as important regulatory cells in autoimmune responses. Abnormalities in the numbers and functions of natural killer T (NKT) cells have been observed in patients with autoimmune diseases as well as in a variety of mouse strains that are genetically predisposed for development of autoimmune diseases. Unlike conventional T cells that recognize peptides in association with major histocompatibility complex (MHC), NKT cells recognize glycolipid antigens presented by the non-polymorphic MHC class I-like protein, CD1d. Recently, we and other groups have demonstrated that administration of glycolipid ligands such as α-galactosylceramide (α-GC) or its sphingosine truncated derivative, OCH suppressed autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), diabetes in NOD mice and collagen-induced arthritis (CIA) by inducing T helper (Th) 2 bias of autoimmune T cells. OCH is a unique ligand to stimulate NKT cells to selectively produce Th2 cytokines whereas α-GC induces both interleukin (IL)-4 and interferon (IFN)-γ, and is more beneficial for treatment of a wide variety of Th1-mediated autoimmune diseases. The lack of polymorphism of CD1d and cross-reactive responses of mouse and human NKT cells to the same ligand indicates that targeting NKT cells with this ligand may be an attractive means for intervening in human autoimmune diseases such as type I diabetes (T1D), multiple sclerosis (MS) and rheumatoid arthritis (RA).

The present review will focus on the potential roles of NKT cells in the pathogenesis of autoimmune diseases and the recent advances in glycolipid therapy for autoimmune disease models. The molecular mechanism of OCH-induced Th2-selective cytokine secretion will also be discussed.

Key Words: NKT cell, CD1, α-galactosylceramide, OCH, autoimmune disease, therapy, Th1/Th2.

INTRODUCTION

Autoimmunity has been studied for more than four decades, but its pathogenesis has remained a mystery. Despite that, potent new biologic therapeutics including cytokines and anti-cytokine reagents show remarkable clinical efficacy in several autoimmune diseases such as MS and RA. However, these drugs have limited value at best, and sometimes are accompanied by serious side effects. Thus drug development for these autoimmune diseases is a fundamental challenge in the 21st century.

It is well-established that central tolerance, the deletion of T cells with high avidity for self-antigens restricts the repertoire of peripheral auto-reactive T cells. However, this process is incomplete. Lymphocytes with lower avidity for self-antigens, or with high avidity for determinants that are not expressed in the thymus (self or foreign), are found in the periphery. The presence of peripheral T cells that react with self-antigens in healthy individuals indicates the existence of physiological regulatory mechanisms that prevent pathological autoimmunity. Such control is referred to as peripheral tolerance, and peripheral tolerance comprises pathways that act directly on auto-reactive cells (intrinsic tolerance: ignorance, anergy, phenotypic skewing) or indirectly through cells such as $CD4^+CD25^+$ T cells and NKT cells [1]. Disruption of these tolerance mechanisms could lead to autoimmune disease. Conversely, maintenance or re-establishment of peripheral tolerance is a therapeutic strategy to restrain destructive autoimmune processes. Thus it seems attractive to induce or stimulate regulatory cells to control harmful autoimmunity [2,3]. Among several different regulatory cells, we would like to focus on targeting NKT cells, because a number of recent studies suggest NKT cells are involved in the pathogenesis of autoimmunity. In addition, several glycolipid ligands can selectively stimulate NKT cells and have been shown to prevent autoimmune disease models.

NKT CELLS AND THEIR ANTIGENS

NKT cells are usually defined as cells co-expressing of the natural killer receptors such as NK1.1 or NKR-P1A (CD161) and a αβ T cell receptor (TCR). Although $NK1.1^+$ TCR^+ lymphocytes are heterogeneous, most NKT cells express an invariant TCRα chain composed of Vα14-Jα18 segments in mice and Vα24-Jα18 segments in humans, which is associated with TCR β chains using a restricted set of Vβ genes. These Vα14 invariant NKT cells recognize glycolipid antigens such as α-GC presented by a nonpolymorphic MHC class I-like molecule, CD1d [4,5]. As little is known about CD1d non-restricted NKT cells or α-GC independent CD1d restricted NKT cells, in this review we focus on the α-GC responsive NKT cells, and "NKT cells" will be used for α-GC responsive NKT cells.

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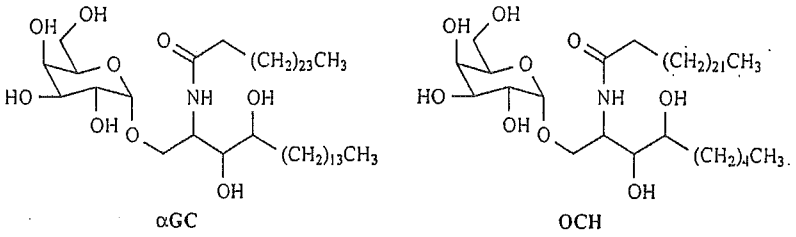

Fig. (1). Structure of α-galactosylceramide (α-GC) and OCH. The α-anomeric conformation of sugar moiety, the configuration of the 2-hydroxyl group on the sugar moiety, 3,4 –hydroxyl groups of the phytosphingoshine are important for NKT cell recognition of α-GC [10]. The OCH analogs has a shorter shpingosine chain.

NKT cells are comprised of two subsets; $CD4^+$ or $CD4^-$ $CD8^-$ (double negative DN). Although the tissue distribution of NKT cells varies, they are most frequent in the liver and bone marrow, and less abundant in the spleen. Whereas human and mouse NKT cells share many characteristics, the frequency is much lower in humans [2]. Moreover, $CD4^+$ and DN NKT cells appear different in terms of cytokine production in humans but not in mice [5,6]. The $CD4^+$ subset of human NKT cells produces both Th1 and Th2 cytokines upon antigen stimulation, whereas the DN subset produces Th1 cytokines and upregulates production of perforin after exposure to cytokines [6].

NKT cells are selected by, and restricted to CD1d. This unique class of antigen-presenting molecules has been highly conserved through mammalian evolution. It is speculated that self glycolipid antigens probably function as activating ligands for NKT cells due to the self-reactivity of NKT cells and the activated memory phenotype of NKT cells isolated from human umbilical-cord blood [7,8] and germ-fee mice [9].

α-GC is a synthetic glycolipid originally isolated from marine sponges *Agelas mauritanius*, and later, a synthetic analog of this compound was developed for experimental studies and clinical trials (Fig. (1)) [10]. α-GC has been shown to be a potent stimulator of both murine and human NKT cells [10-12]. NKT cells respond to sphingolipids substituted with an α-linked galactose or glucose, but not α-linked mannose and sphingolipids containing β-linked galactose or glucose [10]. Sphingolipids containing β-linked sugars resemble common mammalian lipids, whereas α-glycosyl sphingolipids have not been found in normal mammalian tissues. Recently, GD3, a ganglioside expressed on human tumors of neuroectodermal origin has been reported to be recognized by NKT cells [13]. Similar to α-GC, GD3 is not expressed or expressed at low levels on normal tissues.

REGULATION OF CYTOKINE PRODUCTION BY NKT CELLS

NKT cells are characterized by exhibiting a pre-activated phenotype in physiological conditions, being $CD69^+$, $GD62L^{low}$, and $CD44^{high}$. Consistent with the pre-activation status, NKT cells release large amounts of cytokines including IL-4 and IFN-γ promptly upon antigen stimulation and affect the functions of neighboring cell populations such as T cells, B cells, NK cells and dendritic cells (Fig. (2)). [2,5,6]. The mechanisms underlying their rapid cytokine production or their distinct cytokine patterns remain unknown. Recently, Stetson DB *et al.* reported that NKT cells contained 1,000-fold more IL-4 message and 200-fold more IFN-γ message than naive $CD4^+$T cells and levels of H3 acetylation at both the IL-4 and IFN-γ promoters [14]. These chromatin modifications at cytokine genes that correlated with the presence of abundant cytokine mRNAs are similar to differentiated helper T cells such as Th1 or Th2 cells. During differentiation, one set of genes is epigenetically activated and the other is silenced in Th1 or Th2 cells. It is thought that lineage-specific transcription factors such as GATA-3 and T-bet function to maintain and increase the accessibility of one cytokine locus while suppressing or silencing the other in the differentiated cells [15]. Interestingly, NKT cells express both GATA-3 and T-bet allowing hyperacetylation at the IL-4 and IFN-γ promoters (Oki S and Miyake S, unpublished observations).

α-GC induces a variety of cytokines including IFN-γ, IL-2, tumor necrotic factor-α, IL-4 and IL-13 from NKT cells. In contrast, a sphingosine-truncated analogs of α-GC, such as OCH, stimulates NKT cells to preferentially produce IL-4, IL-13. It is important to understand the mechanisms how OCH can stimulate NKT cells to produce Th2 cytokines selectively. IFN-γ production by NKT cells seems to correlate with the stability of glycolipid ligands to bind to the CD1d molecule, and the binding stability correlates with the length of sphingosine chains. Thus OCH binds to CD1d molecule less stably compared to α-GC because of the truncation of sphingosine chain and is therefore not able to sustain TCR stimulation, resulting in preferential production of IL-4 from NKT cells [16]. Given that IL-4 secretion consistently precedes IFN-γ production by NKT cells after TCR ligation, we speculated a critical difference in the upstream transcriptional requirements for the IFN-γ and the IL-4 genes in NKT cells. In support of this speculation, cyclohexamide treatment inhibited the transcription of IFN-γ, but not that of IL-4. In contrast, transcription of both cytokines was abolished by cyclosporine A treatment, indicating that TCR-mediated activation of nuclear factor of activated T cells (NF-AT) is essential for the production of both cytokines. Interestingly, IFN-γ production by NKT cells requires longer TCR stimulation than required for IL-4 when stimulated with immobilized anti-CD3 antibody. TCR stimulation-dependent NF-AT activation is regulated in a manner quite sensitive to change of Ca^{2+} concentration [17]. Thus activated NF-AT might be no longer available for effective IFN-γ transcription due to its quick export from the nucleus after the short duration of TCR stimulation by OCH.

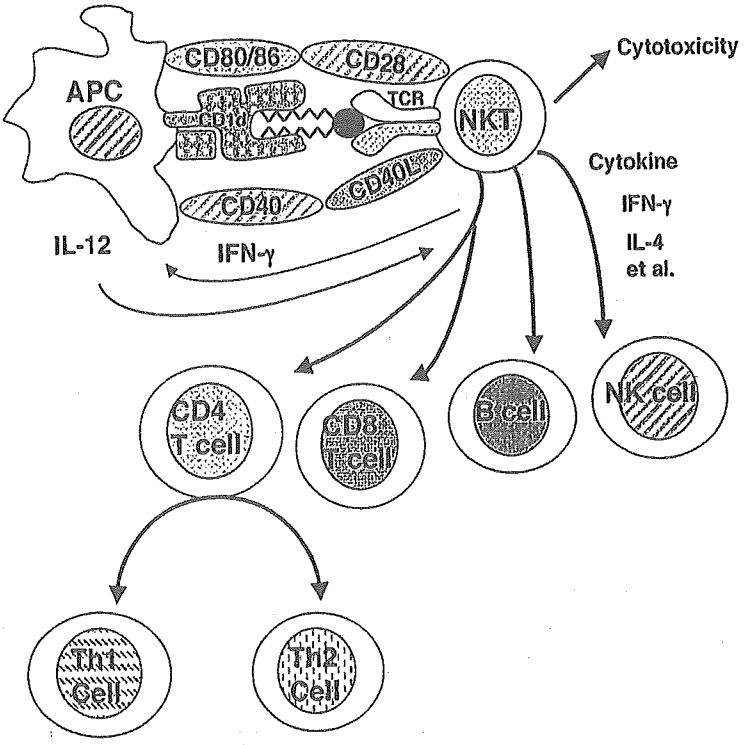

Fig. (2). A model of NK T cell activation and their interactions with other subsets of cells. NKT cells recognize glycolipid ligand presented by CD1d molecule. After stimulation, NKT cells produce a variety of cytokines and exert effector functions. NKT cells might be important for the differentiation of $CD4^+$ T cells into Th1 or Th2 cells, maturation of dendritic cells and activation of B cells and natural killer cells.

Given that *in vivo* administration of soluble OCH and α–GC induces cytokine production by NKT cells within 90 m, stimulation of NKT cells after *in vivo* injection of α–GC or OCH probably occurs without intracellular processing. In fact, when it is presented by antigen presenting cells (APCs) expressing a cytoplasmic tail mutant of the CD1d molecule which is unable to undergo endosomal/lysosomal sorting, the stability of glycolipid antigen binding to CD1d correlated with its length of sphingosine chain. However, when we used APCs expressing wild type CD1d and pulsed antigens for longer time period, the uptake of glycolipids and subsequent endosomal/lysosomal assembly with CD1d, strengthened the interaction of glycolipids with CD1d and abolished the correlation of the binding stability to CD1d and lipid tail length. When we used bone marrow-derived mature dendritic cells as APCs, there was no significant difference between long-term pulsed OCH and α–GC in the ability to induce IFN-γ by freshly isolated NKT cells *in vitro* (Oki S. and Miyake S., unpublished observation).

GLYCOLIPID THERAPIES FOR AUTOIMMUNE DISEASE MODELS

Experimental Autoimmune Encephalomyelitis

EAE is an autoimmune inflammatory disease affecting the central nervous system (CNS) that serves as a model for MS. EAE can be induced in susceptible mouse strains by immunization with CNS proteins or peptides in adjuvant or by the passive transfer of T cells reactive against such CNS antigens. Studies with animal models has suggested that myelin-specific Th1 cells secreting IFN-γ, tumor necrotic factor-α and IL-2 mediate EAE, whereas myelin-specific Th2 cells producing IL-4 and Il-10 play a protective role [18]. Therefore administration of Th2 cytokines to control the disease was considered for clinical use. However, clinical trails of recombinant cytokines, except for IFN-β, have mostly failed because of accompanying side effects. Recently, local delivery of Th2 cytokines, using autoimmune T cells, using hybridomas or fibroblasts transfected with genes encoding anti-inflammatory cytokines was found to be effective in the suppression of EAE [19,20]. However, this strategy seems to be difficult for clinical treatment without major technical advances in introducing particular genes into these cells and in culturing autoimmune T cells. Since NKT cells are known to rapidly invade and accumulate in inflammatory lesions in a manner similar to inflammatory cells and produce cytokines, the stimulation of NKT cells to produce Th2 cytokines would be a powerful strategy to deliver protective cytokines to autoimmune-mediated inflammatory lesions. Nevertheless we observed only a marginal effect of α-GC on the clinical course of EAE induced in C57BL/6 (B6) mice with myelin oligodendrocyte glycoprotein (MOG) derived peptides even though we tried protocols with varying doses of α-GC or different timing of injection [21,22]. Since NKT cells produce both IFN-γ and IL-4 upon stimulation with α-GC, we postulated that α-GC could not prevent EAE because NKT cell-derived IFN-γ would mask the protective effect of the IL-4 simultaneously produced by NKT

cells. We showed several lines of evidence supporting this idea [21]. First, α-GC treatment inhibited EAE induced in IFN-γ knockout mice. Secondly, α-GC treatment augmented the clinical sings of EAE induced in IL-4 knockout mice. Thirdly, blockade of CD86 polarized NKT cells toward a Th2-like phenotype with concomitant suppression of EAE, and activation of APCs by treatment with stimulatory anti-CD40 mAb biased them towards a Th1-like phenotype and exacerbated EAE. As such, EAE could be prevented when ligand stimulation would lead to selective production of Th2 cytokines by NKT cells *in vivo*. Thus we synthesized several analogs of α-GC and found that a sphingosine-truncated analog, OCH, induced selective IL-4 production by NKT cells (Fig. (3)). As expected, administration of OCH prevented development of EAE in both clinical and pathological parameters. The inhibitory effect of OCH was not observed for EAE induced either in NKT cell deficient or IL-4 knockout mice, confirming that IL-4 produced by NKT cells is critical for OCH-mediated suppression on EAE [22].

By contrast, two more reports have shown that α-GC protects mice against EAE when delivered in the immunization protocol (MOG_{35-55} and complete Freund's adjuvant [CFA]) with subsequent multiple intraperitoneal injection or by using a single injection at the day of induction of EAE [23,24]. More recently, Furlan R *et al.* showed that EAE was suppressed only when α-GC was administered at the time of immunization subcutaneously mixed with CFA but not administered intraperitoneally [25]. Although it is not clear the difference among these studies, the role of NKT cells in the pathogenesis or prevention of autoimmunity in CNS may depend on the stage of disease and the associated cytokine milieu, the timing or the route of administration. These parameters are critical to modulate diseases.

In addition to B6 mice, SJL mice are highly susceptible to EAE and EAE induced by immunization with proteolipid protein derived peptides $PLP_{139-151}$ is used as a remitting-relapsing MS model. In the context of NKT cells, SJL mice have been reported to be markedly diminished in number and cytokine production upon activation [26]. Singh AK *et al.* reported that SJL mice responded poorly to treatment with α-GC [24]. When SJL mice were treated with α–GC, the morbidity and mortality were exacerbated although the onset of disease was delayed. By contrast, a multiple injection of OCH protected SJL mice against EAE (Miyake S and Yamamura T, unpublished observation). Furthermore, OCH protected SJL mice against the relapse of EAE, suggesting

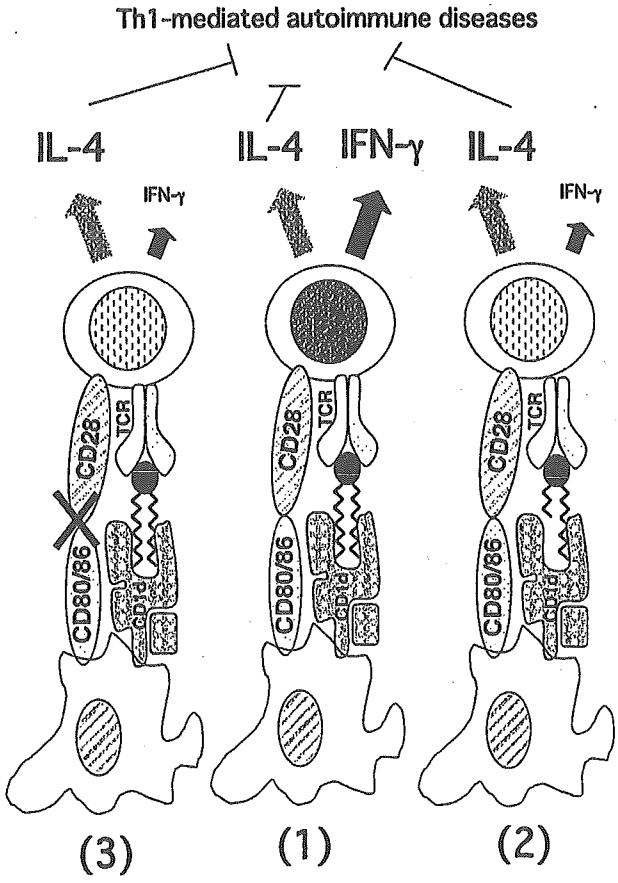

Fig. (3). Modulation of NKT cell cytokine production by an altered ligand or by co-stimulator blockade. 1) α-GC stimulates NKT cells to produce both anti-inflammatory (e.g. IL-4 and IL-10) and pro-inflammatory (e.g. IFN-γ) factors. This response can be modified by 2) stimulation with an altered ligand such as OCH or 3) stimulation in the absence of CD28/B7.2 co-stimulation. These modifications are potentially important therapeutic approach to suppress Th1-mediated autoimmune diseases.

that OCH holds possibilities as a therapeutic agent to prevent relapses for MS.

Glycolipid Therapy for Collagen-induced Arthritis

RA is an autoimmune disease characterized by persistent inflammation of joints resulting progressive destruction of cartilage and bone. Although its precise etiology is not clearly understood, cumulative evidence suggests that Th1 cells exacerbate disease, whereas Th2 cells suppress arthritis [27]. Given that NKT stimulation with OCH suppressed Th1-mediated diseases such as EAE, OCH might be an effective therapeutic reagent for CIA which serves as an animal model for RA. We have demonstrated that OCH administration inhibited the clinical course of CIA induced in B6 mice by immunization with the chicken type II collagen [28]. Histological analysis revealed that OCH treatment protected against infiltration of inflammatory cells and destruction of cartilage and bone. The suppressive effect of OCH was not observed for CIA induced either in CD1d knockout mice or in Jα18 knockout mice deficient in NKT cells. We also observed OCH suppressed CIA induced in DBA/1J mice immunized with bovine type II collagen. Moreover, injection of OCH strongly suppressed CIA in SJL mice even though these mice have defects in numbers and functions of NKT cells, and even after the arthritis had already developed. By contrast, administration of α-GC didn't suppress arthritis in any of these three models. Suppression of arthritis was associated with the elevation of IgG1:IgG2a ratio indicating the Th2 bias of type II collagen-reactive T cells. Injection of neutralizing antibody to either IL-10 or IL-4 reversed the beneficial effect of OCH treatment. These results imply that IL-10 and IL-4 are critical in the OCH-mediated suppression of CIA and are consistent with our idea that OCH modulated CIA by stimulating the production of Th2 cytokines from NKT cells although the source of IL-10 remains to be elucidated. Since OCH seems a potential therapeutical tool to suppress arthritis, the role of NKT cells in the natural course of arthritis should be clarified in the future.

Glycolipid Therapy for Autoimmune Diabetes in NOD Mice

Nonobese diabetic (NOD) mice develop a spontaneous autoimmune diabetes similar to the human T1D. Autoimmune destruction of β cells is preceded by infiltration of pancreatic islets by macrophages, B cells and T lymphocytes [29,30]. Many studies have indicated that Th1 type $CD4^+$ cells and $CD8^+$ T cells have been implicated in the development of diabetes in the NOD mouse. In parallel with these effector cells, the regulatory cells including NKT cells have been suggested to inhibit the development of diabetes. Although the mechanisms of suppressive effect of these regulatory T cells are not fully understood, it is believed that an imbalance between autoreactive effector T cells and regulatory T cells may trigger the development of destructive insulitis and diabetes [28].

Studies have indicated that NOD mice were deficient in the number and function of NKT cells [31]. Although the correlation between a defect in NKT cells and the susceptibility of diabetes in NOD mice is still debated [3,32,33], the putative involvement of NKT cells in the control of islet β-cell reactive T cells in NOD mice was suggested by prevention of diabetes following infusion of NKT cell enriched thymocytes preparations [34] and by the increase of NKT cells in Vα14Jα281 transgenic NOD mice [35].

Several recent papers investigated the effect of treating NOD mice with α-GC [33,35-38]. When started around three or four weeks of age, repeated injections at least once a week delayed the onset and reduced the incidence of diabetes. After treatment, splenocytes from NOD mice produced a greater amount of IL-4 in response to islet antigens and the IgG1/IgG2a (Th2/Th1) ratio of anti-GAD antibody increased. Thus it appears that the mechanism of protection is similar to that observed by increasing the numbers of NKT cells in NOD mice and by α–GC treatment in other autoimmune disease models such as EAE and CIA. This effect was auto-antigen specific as no difference was observed in the immune response to ovalbumin [36]. However, the mechanism in which glycolipid treatment induces an auto-antigen specific switch in the immune response of NOD mice is unclear. We also observed the protective effect of OCH treatment in NOD diabetic mice in addition to α-GC treatment. The protective effect for insulitis by OCH was more profound compared to that by α-GC [56].

GLYCOLIPID THERAPY FOR MOUSE MODELS OF SYSTEMIC LUPUS ERYTHEMATOSUS

It has been reported that a selective reduction in $NK1.1^+$ T cells precedes the development of autoimmunity in MLR lpr/lpr mice. Mieza MA *et al.* also found a decrease in the expression of invariant Vα14 TCR mRNA of NKT cells before the onset of lymphocyte accumulation and autoimmune disease in MRL lpr/lpr mice, C3H gld/gld and NB/W F1 mice when compared to control mice [39]. Recently, Zeng D *et al.* demonstrated that treatment of NZB/W F1 mice with anti-CD1d monoclonal antibody augmented Th2-type responses, increased serum levels of IgE, decreased levels of IgG2a and IgG2a anti-double-stranded DNA (dsDNA) antibodies, and ameliorated lupus [40]. They also showed that multiple injection of α-GC induced an enhanced Th1-type response and exacerbated lupus associated with decreased serum levels of IgE and increased levels of IgG2a and IgG2a anti-ds DNA antibodies. This exacerbation of disease was associated with reduced IL-4 and tumor necrotic factor-α production and expansion of marginal zone B cells. These results suggested that activation of NKT cells augmented Th1-type responses and autoantibody production that contribute to lupus development in NZB/W F1 mice. In contrast, Yang JQ *et al.* reported that pristine-induced lupus nephritis was accelerated when induced in CD1d deficient mice [41]. They also demonstrated that repeated injection of α-GC resulted in the expansion of NKT cells and ameliorated dermatitis in MRL lpr/lpr mice [42]. Thus they postulated that NKT cells may play a protective role in lupus models. Since lupus models are not simply explained by only Th1-mediated or Th2-mediated pathology, the complexity of these models may explain the differences in results in these studies.

NKT Cells in Human Autoimmune Diseases

Multiple Sclerosis

MS is an autoimmune demyelinating disease of the CNS. Illes Z *et al.* reported a reduction in Vα24Jα18 cells among $Vα24^+$ cells from the peripheral blood of patients with MS compared to healthy subjects using single-strand conformation polymorphism method to detect TCR gene rearrangements [43]. Van der Vliet *et al.* showed a decrease in the number of NKT cells by screening of $Vα24^+Vβ11^+$ cells in the blood [44]. Araki M *et al.* demonstrated that DN NKT cells in the periphery were greatly reduced in remission whereas the reduction of $CD4^+$ NKT cells was marginal [45]. Furthermore $CD4^+$ NKT line cells expanded from MS in remission produced a larger amount of IL-4 than those from healthy subjects or from MS in relapse. Therefore, we speculated that the Th2 bias of $CD4^+$ NKT cells may play a role in the regulation of Th1 type autoantigen reactive T cells. In contrast, Gausilng *et al.* did not find a significant difference in the number of DN $Vα24^+$ NKT cells in PBL between from MS patients and from healthy controls [46]. The basis for the discrepancy between the number of NKT cells among these studies is not clear. Considering that the proportion of Vα24Jα18 T cells in normal individuals varies among studies, it may not be easy to compare these studies.

Type I Diabetes

Studies of the frequency of human NKT cells in PBMCs in patients with T1D have had conflicting results. In initial studies, Wilson B *et al.* studied identical twin/triplet sets discordant for disease, and reported that diabetic siblings have lower frequencies of DN Vα24Jα18 T cells in their peripheral blood than non-diabetic siblings [47]. In addition, Kukreja AG *et al.* showed a reduction in the number of NKT cells in newly diagnosed patients [48]. However, more recent papers reported unaltered or increased NKT cells in recent-onset patients with type I diabetes [49,50]. Wilson B *et al.* also showed that DN Vα24Jα18 T cell clones isolated from diabetics had an impaired ability to produce IL-4 [47]. In contrast, Lee PT *et al.* reported IL-4 production by NKT cells was similar among these groups as assessed by intracytoplasmic staining following short-term PMA and ionomycin stimulation [49]. At this stage, it is hard to interpret the discrepancies between these results, since the methods for detecting NKT cells and the functional assays used differ between these studies.

Systemic Autoimmune Disease

Sumida and colleagues found that αβ+ DN T cells were increased in Scleroderma patients and that there was oligoclonal expansion of $Vα24^+TCR^+$ cell among these cells [51]. Although the invariant Vα24Jα18 T cells were dominant among these cells from healthy donors, invariant Vα24Jα18 T cells were replaced by clones with other Vα24 TCR^+ cells in Scleroderma patients. In addition, Maeda *et al.* have reported the expansion of non-invariant Vα24 TCR^+ cells but not Vα24Jα18 T cells in the synovium of RA patients [52]. Similar to this study, these authors observed the expansion of non-invariant Vα24 TCR^+ clones in patients with active SLE [53, 54]. Furthermore, following predonisolone therapy, Vα24Jα18 T cells increased among Vα24 TCR^+ cells. The recovery of Vα24Jα18 T cells in patients with predonisolone therapy was also observed among patients with MS (Araki M and Yamamura T, unpublished observation). Kojo S *et al.* and other groups investigated the number of NKT cells using Vα24 and Vβ11 mAb to detect NKT cells in patients with several different autoimmune diseases, including SLE, Scleroderma and RA [43, 55]. They found lower numbers of $Vα24^+Vβ11^+$ NKT cells in the peripheral blood than controls. Kojo S *et al.* also showed in this study that half of the patients with autoimmune disease responded to α-GC in culture.

PROSPECTS FOR GLYCOLIPID THERAPY FOR AUTOIMMUNE DISEASES

It remains unclear whether the defect in NKT cells is causal for autoimmune disease or occur as a secondary consequence of the autoimmune process. However, given the efficacy of OCH and α-GC in mouse models, stimulation of NKT cells with glycolipid antigens seems to be an attractive strategy for the treatment of autoimmune diseases. Although several studies have shown that administration of α-GC caused liver damage, the hepatotoxicity was minimal in Phase I trials of α-GC for patients with cancer. Given the lack of severe toxicity in humans, it seems reasonable to use glycolipids for the prevention or therapy of selected human autoimmune disorders. α-GC has been shown to exacerbate EAE, depending on the strain of mouse and stage of disease tested and to have only a marginal effect on CIA. In this situation, treatment with OCH might be preferable to α-GC for Th1-mediated diseases such as MS, type I diabetes and RA, as OCH elicits a predominantly IL-4 response rather than IFN-γ in contrast to α-GC, which might afford greater protection from EAE and MS.

Both rodent and human NKT ells have been reported to recognize α-GC in the context of CD1d. OCH also stimulates human NKT cells, particularly $CD4^+$ NKT cells, and OCH stimulation induces more Th2 cytokine production from NKT cells compared to α-GC stimulation (Araki M and Yamamura T, unpublished observation). The evolutionary conservation and the homogeneous ligand specificity of NKT cells allow us to apply a glycolipid ligand like OCH for the treatment of human disease without considering species barrier or genetic heterogeneity of humans.

CONCLUSION

In this review, we have discussed the supporting data for the role of NKT cells in the regulation of autoimmune diseases. Ligand stimulation of NKT cells is an attractive strategy for prevention or treatment of autoimmune diseases. However, the mechanisms by which NKT cells exert their immunoregulatory functions are still largely unknown and a number of questions require further investigation including the mechanism to recruit NKT cells and control its functions at inflammatory sites and the interaction of other subsets of cells. To clarify the nature of natural ligands for NKT cells is one of the major questions and it could be an interesting natural source of useful ligands for CD1-restricted regulatory cells.

ABBREVIATIONS

NKT = Natural killer T

MHC = Major histocompatibility complex

α-GC = α-galactosylceramide

EAE = Experimental autoimmune encephalomyelitis

CIA = Collagen induced arthritis

Th = T helper

IL = Interleukin

IFN = Interferon

T1D = Type 1 diabetes

MS = Multiple sclerosis

RA = Rheumatoid arthritis

TCR = T cell receptor

DN = Double negative

NF-AT = Nuclear factor of activated T cells

APC = Antigen presenting cell

CNS = Central nervous system

B6 = C57BL/6

MOG = Myelin oligodendrocyte glycoprotein

CFA = Freund's complete adjuvant

NOD = Nonobese diabetic

dsDNA = Double-stranded DNA

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Lecture

解　説

NK T細胞を標的とした自己免疫疾患治療法*

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Key Words：NK T cell, glycolipid ligand, autoimmune diseases

NK T細胞とは

NK T細胞は，NKマーカーを有するT細胞の総称であり，いくつかのサブポピュレーションがあることが知られている．その中で，T細胞受容体(TCR) α鎖に可変性のないinvariant鎖(マウスではVα14Jα18，ヒトではVα24Jα18)を発現する細胞がもっとも解析が進んでおり，自己免疫との関連も研究されているので，本稿ではNK T細胞をiVα14(ヒトではVα24)NK T細胞と定義して概説する[1)~3)]．Vα14NK T細胞は，限られた*Vβ*遺伝子(マウスではVβ8.2，Vβ7，Vβ2，ヒトではVβ11)と会合するため，TCRの可変性が乏しく，また，主要組織適合遺伝子複合体(MHC)クラスⅠ類似のCD1d分子に提示された糖脂質を抗原として認識する．CD1分子はβ2マイクログロブリンと非共有結合したヘテロ二量体として細胞表面に発現し，MHCクラスⅠ分子に類似している(図1)．CD1ファミリーには，ヒトではグループ1 CD1(CD1a, b, c)とグループ2 CD1(CD1d)が知られているが，マウスではCD1d分子のみが存在する．CD1分子は，MHC分子と異なり多様性がないため，同一種内では共通である．CD1d分子は，抗原結合溝はきわめて疎水性である．抗原結合溝が疎水性であること，NK T細胞の発生が抗原関連トランスポーター(TAP)非依存性であること，ほかのCD1ファミリー分子が結核菌細胞壁の構成脂質であるミコール酸やレプラ菌の細胞壁糖脂質であるリポアラビノマンナン(LAM)を抗原提示することなどから，CD1dに抗原提示を受けるNK T細胞も，蛋白ではなく糖脂質をリガンドとして認識すると考えられた．直接的な証明としては，NK T細胞のリガンドとして，谷口らが海綿の成分であるα-ガラクトシルセラミド(α-GC)がNK T細胞のリガンドとなることを発見したことである[4)]．α-GCは哺乳類の体内に存在することは証明されていないので，NK T細胞の生体内での抗原が何かについては，研究が続けられている．ヒトの臍帯血中のNK T細胞や，germ freeマウスのNK T細胞も，すでにメモリーマーカーが陽性であることから，なんらかの自己抗原を認識しているのではないかと考えられている．これまで，内因性抗原としては，glycosylphosphatidylinositol(GPI)，ガングリオシドGD3, phosphoethanolamine(PE)などがリガンドとして報告されている[5)~7)]．また，外来抗原としては，Leishmania由来のglycoinositol phospholipids(GIPLs)やlipophosphoglycan(LPG)，Sphingomonas由来のglycosphingolipid(GSL)などが報告されている[8)]．機能的な特徴としては，TCRを介した刺激によりIL-4, IFN-γを含む多くのサイトカインを短時間で大量に産生することから，感染症，癌免疫，移植などさまざまな場面でその免疫調節機能が注目されている．とくに自己免疫疾患においては，α-GCやその誘導体であるOCH(図1)などの糖脂質抗原を用いて，NODマウスにおける糖尿病，実験的自己免

* Therapeutic potential of targeting natural killer T cells in the suppression of autoimmune diseases.

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