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NKG2D in NK and T Cell-Mediated Immunity

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One of the best characterized NK cell receptors is NKG2D, a highly conserved C-type lectin-like membrane glycoprotein expressed on essentially all NK cells, as well as on $\gamma\delta$-TcR$^+$ T cells and $\alpha\beta$-TcR$^+$ CD8$^+$ T cells, in humans and mice. Here we review recent studies implicating NKG2D in T cell and NK cell-mediated immunity to viruses and tumors, and its potential role in autoimmune diseases and allogeneic bone marrow transplantation.

KEY WORDS: NK cells; NKG2D; DAP10; RAE-1; MICA; autoimmunity; bone marrow transplantation; T cell.

INTRODUCTION

NKG2D is an activating receptor expressed on NK cells, CD8$^+$ T cells and $\gamma\delta$-TcR$^+$ T cells (1–3). NKG2D, a type II transmembrane glycoprotein expressed as a disulfide-bonded homodimer, itself has no signaling motifs in its intracellular region (4). Instead, NKG2D associates noncovalently with the DAP10 adapter protein in mouse and human NK cells and T cells (2, 5). In mice, an NKG2D isoform generated by alternative splicing can also associate with the DAP12 adapter protein in activated mouse NK cells (5, 6), whereas human NKG2D pairs only with DAP10 and not DAP12 (7). Association between NKG2D and its adapter proteins occurs by interactions between their transmembrane domains through a salt bridge formed by opposing charged residues (7, 8), and the NKG2D–DAP10 receptor complex is expressed on the cell surface as a hexamer with each subunit of NKG2D noncovalently associated with two DAP10 disulfide-bonded homodimers (9) (Fig. 1). The cytoplasmic domain of DAP10 has a YxxM motif, which recruits the p85 phoshoinositide-3 (PI3) kinase subunit and Grb2 (2, 11). DAP12 has an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain, which recruits and activates the Syk and ZAP70 protein tyrosine kinases (12). Therefore, in mouse and human NK cells, NKG2D-dependent activation uses the PI3 kinase pathway, and in activated mouse NK cells the ITAM-induced Syk/ZAP70 pathway.

NKG2D binds to a family of cell surface glycoproteins with structural homology to MHC class I proteins (reviewed in 13–15) (Fig. 2). In humans, the NKG2D ligands are MHC class-I-related chain (MIC) A and MICB (17) and the UL-16 binding protein (ULBP) (also called RAET1) family of glycoproteins (18–21). In mice, the retinoic acid early inducible-1 (Rae-1) family of proteins, H60, and murine ULBP-like transcript 1 (MULT1) were identified as high-affinity ligands for NKG2D (22–24). The *MICA*, *MICB*, *ULBP* (*RAET1*), *RAE-1*, and *H60* genes are polymorphic, such that within the human population or in different strains of mice there is considerable diversity. Although MULT1 mRNA (but not protein) was detected ubiquitously (24), other NKG2D ligands in mice are mostly silent in normal, healthy adult tissues (13). In mice, RAE-1 is frequently expressed on tumors and on cells during viral and bacterial infections (22, 23, 25–29). The MICA/B ligands of NKG2D are known to be stress-inducible molecules (30). Cell surface expression of MICA and MICB, which are under the control of a heat shock promoter, can be induced by cellular stress, including heat shock, transformation, or viral or bacterial infections (30–33). In addition, recent studies have shown that the *RAE-1* and *UPBP* genes can be induced by the pathways involved in cellular responses to DNA damage (34). Interestingly, expression of the NKG2D ligands has also observed in human and mouse autoimmune diseases (35, 36).

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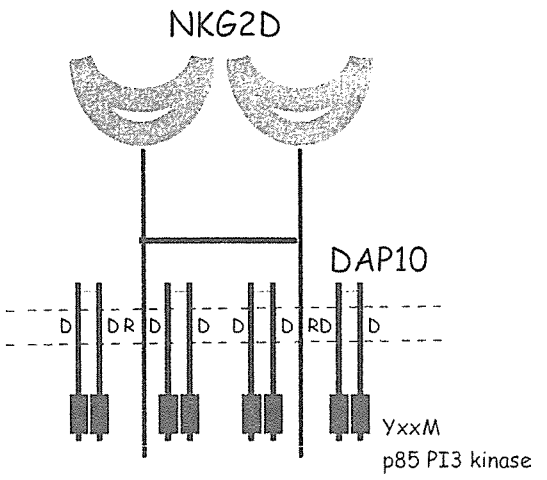

Fig. 1. Schematic representation of the NKG2D-DAP10 receptor complex. Two NKG2D subunits, each a type II transmembrane-anchored glycoprotein of the C-type lectin-like superfamily, are disulfide-bonded to form a homodimer. Stable expression at the cell surface requires noncovalent association with the DAP10 adapter protein (10). DAP10 is a type I transmembrane-anchored glycoprotein with a minimal extracellular region and a small cytoplasmic domain with the motif YINM, which upon phosphorylation binds and activates the p85 subunit of PI3-kinase. DAP10 is also expressed as a disulfide-bonded homodimer, and two DAP10 homodimers associate noncovalently with each NKG2D homodimer, resulting in a hexameric receptor complex (9).

NKG2D IN VIRAL IMMUNITY

Mouse and human cytomegalovirus (CMV) have evolved elaborate evasion mechanisms to avoid recognition by NKG2D-bearing NK cells and T cells (26, 27, 37–40). NKG2D ligands are expressed on MCMV-infected peritoneal macrophages (26) and MIC is up-regulated in lung tissues of HCMV-infected humans (32). In addition, MCMV titers were decreased in BALB/c mice infected with MCMV stains lacking the viral *m152*, *m145*, and *m155* genes responsible for preventing expression of the mouse RAE-1, MULT1, and H60 proteins, respectively, on infected cells (26, 27, 37). HCMV encodes the UL16 protein that intracellularly retains three of the six human NKG2D ligands (ULBP1, ULBP2, and MICB) (18, 38–41). When fibroblasts are infected with a UL16-deletion HCMV, human NK cells kill these cells more efficiently than fibroblasts infected with wild-type HCMV (38). Moreover, certain alleles of MICA that are common in the human population are not affected by HCMV, suggesting that the *MICA* gene may be under selective pressure to avoid the NKG2D evasion mechanisms of the virus (42). MICB has been detected on human macrophages infected with influenza A or Sendai virus, by a mechanism at least partially dependent on virus-induced IFNα production (43). A role for NKG2D in mouse hepatitis virus is suggested by studies of Dandekar *et al.* (29), who

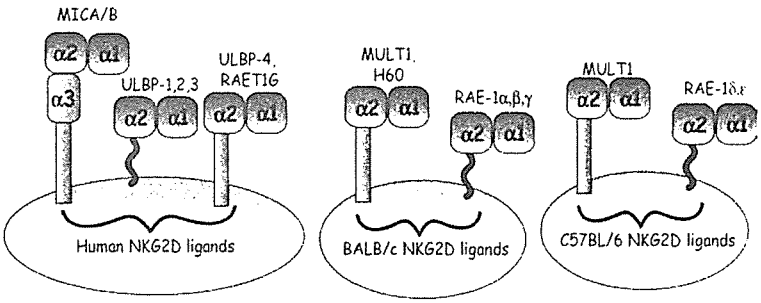

Fig. 2. Schematic representation of human and mouse NKG2D ligands. MICA and MICB are transmembrane-anchored type I glycoproteins with an α1, α 2, and α 3 domain. Within the ULBP (RAET1) family, which are type I glycoproteins with an a1 and a2 domain, ULBP-1, -2, and -3 are GPI-anchored to the membrane, whereas ULBP-4 (RAET1E) and RAET1G are transmembrane-anchored. *MICA*, *MICB*, and the *RAET1* genes are polymorphic; 60 alleles of MICA have been identified and 25 alleles of MICB have been described. The mouse *RAE-1* genes, which encode GPI-anchored type I glycoproteins, are also polymorphic; C57BL/6 mice possess RAE-1δ and RAE-1ε, whereas BALB/c and 129/J mice possess RAE-1α, β, and γ. It is presently unknown where RAE-1δ and RAE-1ε are allelic variants of RAE-1α, β, and γ, or alternatively are distinct loci. BALB/c, but not C57BL/6, express a functional *H60* gene (16). MULT1, encoding a transmembrane-anchored type I glycoprotein, is expressed by both C57BL/6 and BALB/c mice; allelic polymorphisms of MULT1 have not yet been identified. All these NKG2D ligands have α1 and α2 domains with structural homology to MHC class I, and MICA and MICB have an α3 domain—yet none of the NKG2D ligands associate with β2-microglobulin or bind peptides.

have shown that the pathology caused by MHV infection of the central nervous system is due, in part, to an NKG2D-dependent mechanism mediated by $\gamma\delta$-TcR$^+$ T cells. Collectively, these studies indicate the importance of NKG2D in immune responses against viral infection and the countermeasures taken by viruses to prevent this surveillance.

NKG2D IN TUMOR IMMUNITY

Because tumors develop from self-cells, in general tumors are poorly immunogenic and often are not recognized efficiently by the adaptive immune system. However, the NKG2D ligands are frequently expressed on a substantial number of human and mouse tumors, and, therefore, could potentially serve as "danger signals" to alert the immune system to the existence of these abnormal cells (22, 23, 31). Mouse tumors expressing endogenous NKG2D ligands or transfected with cDNA encoding NKG2D ligands are sensitive to NKG2D-dependent NK cell-mediated cytotoxicity *in vivo* and *in vitro* (3, 22, 23, 44–47). Similarly, human NK cells and $\gamma\delta$-TcR$^+$ T cells can efficiently kill tumors bearing NKG2D ligands (1, 31, 48). In some cases, expression of NKG2D ligands on tumors can augment the generation of tumor-specific T cells (45, 49). Studies have also demonstrated that tumors expressing NKG2D ligands are more responsive to immunotherapy with IL-2, IL-12, and IL-21 compared with tumors lacking ligands for this activating NK receptor (46, 47, 50). Thus, NKG2D ligands expression induces NKG2D-dependent tumor elimination, which potentially may be exploited for therapeutic benefit.

However, the presence of NKG2D ligands on numerous human and mouse tumors means that these tumors have managed to escape from detection or elimination by the immune system *in vivo*. Several mechanisms may account for the emergence of these NKG2D ligand-bearing tumors. Groh and colleagues reported that some cancer patients have high concentrations of soluble MIC proteins in their serum, and this correlated with down-regulation of NKG2D receptor expression on the CD8$^+$ T cells in these individuals (51). Soluble NKG2D ligands may be generated by alternative splicing of certain *MIC* genes, resulting in the generation of transcripts lacking a transmembrane and cytoplasmic domain that can produce a soluble MIC protein. Alternatively, the soluble NKG2D ligand proteins may originate by cleavage from the cell surface of tumor cells by metalloprotease (52, 53). Chronic exposure of NK cells or CD8$^+$ T cells to cell surface or soluble NKG2D ligands leads to modulation of NKG2D and subsequent functional impairment of NKG2D-dependent activation (51, 54–57). Thus, prolonged exposure to ligands may desensitize NK cells rendering them functionally anergic. Antitumor immunity may also be impaired by TGF-β1 secreted by tumors, which causes the down-regulation of NKG2D on NK cells and CD8$^+$ T cells in the tumor microenvironment (58, 59). This has implications in the context of tumor development because secretion of soluble NKG2D ligands by tumors or prolonged exposure of NK cells to ligand-bearing tumors or TGF-β1 may render them dysfunctional, which may contribute to tumor escape from NK cell and CD8$^+$ T cells immune surveillance. A role for NKG2D in surveillance against primary tumorigenesis was revealed by the finding that mice exposed to chemical carcinogens developed fibrosarcomas at a higher incidence when treated with an neutralizing anti-NKG2D monoclonal antibody (50) or when RAE-1 was constitutively overexpressed as a transgene in the carcinogen-treated mice (56).

NKG2D IN BONE MARROW REJECTION

Although beneficial in host protection against infectious diseases and tumors, mouse NK cells can reject bone marrow cell grafts (60–62). Interestingly, F1 recipients accept parental skin grafts, but reject bone marrow cell grafts—a process called F1 hybrid resistance (63, 64). Initially, the hypothesis proposed to explain F1 hybrid resistance was rejection based on the expression of hybrid histocompatibility (Hh) antigens on parental bone marrow cells that were not expressed in the F1 hybrid mice. Genetic mapping studies suggested that at least in some mouse strains the genes regulating the Hh antigens localized to the H-2S-H2-D region (65). Recently, the ability of NK cells to recognize and reject parental BM cells has been explained, in part, by the lack of inhibitory Ly49 receptors specific for parental H-2 proteins on a subset of NK cells in the F1 recipient (66–68). Thus, a subset of NK cells in the F1 recipient lacking inhibitory receptors for the parental bone marrow cells might eliminate these parental bone marrow grafts because they are unable to recognize the parental H-2 antigens. However, the activating NK cell receptors on the NK cells in the F1 recipient that initiate the attack against the parental bone marrow cells have not been defined.

Parental BALB/c or C57BL/6 bone marrow cells are able to repopulate in NK cell-depleted (C57BL/6 $\times$ BALB/c) irradiated F1 mice. In this situation, we found that one of NKG2D ligands, RAE-1, is expressed on repopulating bone marrow cells, predominantly myeloid progenitor cells, from BALB/c mice but not C57BL/6 mice (55). Treatment with anti-NKG2D

mAb prevented rejection of parental BALB/c bone marrow in (C57BL/6 × BALB/c) F1 recipients. Thus, NKG2D is involved in F1 hybrid resistance against certain mouse strains such as BALB/c. However, NKG2D blockade had no effect on the rejection of C57BL/6 parental bone marrow grafts in (C57BL/6 × BALB/c) F1 recipients. These results indicate that both NKG2D-dependent and NKG2D-independent mechanisms are operative in NK cell-mediated bone marrow rejection and that these are genetically regulated. In humans, NKG2D ligands are also expressed on myeloid progenitor cells (69).

Furthermore, we found that bone marrow from C57BL/6 expressing a RAE-1ε transgene under an actin promoter were rejected by syngeneic mice (55). These data indicate that NK cells are able to reject syngeneic C57BL/6 bone marrow cells when these otherwise normal bone marrow cells expressed sufficient amounts of an NKG2D ligand. Therefore, if other stem cells or tissues up-regulate expression of NKG2D ligands after transplantation, NKG2D may contribute to graft rejection in immunocompetent hosts.

NKG2D IN AUTOIMMUNITY

Recent studies suggest that NK cells and T cells expressing NKG2D participate in autoimmune diseases (35, 36, 54, 70–75). Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease in which insulin-producing β cells are destroyed by autoreactive T cells (reviewed in 76). NOD mice spontaneously develop IDDM, and serve as an animal model of this disease. We recently observed that RAE-1 proteins are aberrantly expressed on the prediabetic islet cells in NOD mice, and the autoreactive $CD8^+$ T cells infiltrating pancreas expressed NKG2D (36). Treatment with anti-NKG2D mAb during the prediabetic stage completely prevented diabetes by impairing the expansion and function of autoreactive $CD8^+$T cells. These findings demonstrate that NKG2D is essential for disease progression and suggest a new therapeutic target for autoimmune diabetes. It has been reported that NK cells are involved in autoimmune diabetes; depletion of NK cells delayed the onset of diabetes in BDC2.5 transgenic mice (expressing a TcR against an undefined pancreatic autoantigen) on the C57BL/6.H-2g7 genetic backgrounds (70). These data suggest that NK cells and T cells expressing NKG2D are involved in the development of autoimmune diabetes.

In humans, MICA has been detected on synoviocytes in the joints of patients with rheumatoid arthritis (RA), accompanied by the presence of an unusual subset of $CD4^+$ T cells that lack CD28 but express NKG2D (35). MICA is induced on proliferating RA synoviocytes and MICA may contribute to joint disease perpetuation and progression by costimulation of $CD4^+CD28^-$ T cell cytokine production and proliferation. Similarly, overexpression of MICA has been detected in the intestine of patients with celiac disease, and it has been suggested that IL-15 activated intestinal $CD8^+$ cytotoxic T lymphocytes, which express NKG2D, may attack MICA-bearing tissues (71, 73, 75). Collectively, these studies implicate NKG2D in autoimmune diseases in humans and mouse model systems, suggesting this pathway as an attractive target for new therapeutics.

CONCLUSIONS

NKG2D is emerging as a central player in innate and adaptive immunity—serving a fundamental role in the surveillance against microbial infection and cancer. As with many other elements of the immune system, this pathway may also be deleterious by serving as a barrier to tissue transplantation and in autoimmune diseases.

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TROY and LINGO-1 expression in astrocytes and macrophages/microglia in multiple sclerosis lesions

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Nogo constitutes a family of neurite outgrowth inhibitors contributing to a failure of axonal regeneration in the adult central nervous system (CNS). Nogo-A is expressed exclusively on oligodendrocytes where Nogo-66 segment binds to Nogo receptor (NgR) expressed on neuronal axons. NgR signalling requires a coreceptor $p75^{NTR}$ or TROY in combination with an adaptor LINGO-1. To characterize the cell types expressing the NgR complex in the human CNS, we studied demyelinating lesions of multiple sclerosis (MS) brains by immunohistochemistry. TROY and LINGO-1 were identified in subpopulations of reactive astrocytes, macrophages/microglia and neurones but not in oligodendrocytes. TROY was up-regulated, whereas LINGO-1 was reduced in MS brains by Western blot. These results suggest that the ternary complex of NgR/TROY/LINGO-1 expressed on astrocytes, macrophages/microglia and neurones, by interacting with Nogo-A on oligodendrocytes, might modulate glial–neuronal interactions in demyelinating lesions of MS.

Keywords: LINGO-1, macrophages/ microglia, multiple sclerosis, reactive astrocytes, TROY

Introduction

Axonal damage beginning at the early stage of multiple sclerosis (MS) is responsible for permanent neurological deficits and progression of clinical disability [1]. Although the underlying molecular mechanism remains unknown, the degree of inflammatory demyelination correlates with the extent of axonal damage, suggesting an involvement of proinflammatory mediators in inducing axonal degeneration [2]. However, the alternative possibility could be proposed that axonal regeneration is severely impaired in MS lesions, because an accumulation of glial scar and neurite growth inhibitors provide a non-permissive environment for regrowth of damaged axons [3].

Nogo constitutes a family of myelin-associated inhibitors of axonal regeneration, composed of three isoforms named A, B and C, all of which share a C-terminal 66-amino-acid segment named Nogo-66 [4]. Nogo-A, the longest isoform, is expressed exclusively on oligodendrocytes and myelin sheath and a subpopulation of neurones [5]. Nogo-66 as well as oligodendrocyte-myelin glycoprotein (OMgp) and myelin-associated glycoprotein (MAG) binds to the Nogo receptor (NgR), a glycosylphosphatidylinositol (GPI)-anchored membrane protein expressed chiefly on a subpopulation of neurones and their axons, including cerebral cortical pyramidal neurones and cerebellar Purkinje cells [6]. NgR transduces inhibitory signals via a coreceptor $p75^{NTR}$ that acts as a displacement factor releasing a small GTP-binding protein RhoA, an

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intracellular regulator of the cytoskeleton, from the Rho GDP dissociation inhibitor [6,7]. Neurones lacking $p75^{NTR}$ neither show RhoA activation nor exhibit neurite growth inhibition in the presence of myelin components, suggesting a key role of $p75^{NTR}$ in the NgR signal transduction *in vitro* [6]. However, the role of $p75^{NTR}$ remains unclear in the adult central nervous system (CNS) *in vivo* where NgR is identified in many neurones that exhibit little or no $p75^{NTR}$ expression [8].

Recently, two novel components of the NgR signalling complex, named TROY and LINGO-1, have been identified. TROY is a type I membrane protein belonging to the tumour necrosis factor receptor superfamily that substitutes for $p75^{NTR}$ in the NgR signalling complex to activate RhoA following exposure to myelin-associated neurite growth inhibitors [9,10]. TROY is strongly expressed during development in neural stem cells located in the ventricular and subventricular zones, and is more broadly expressed in various neurones in the adult rodent CNS than $p75^{NTR}$ [9–12]. Overexpression of a truncated form of TROY lacking its intracellular domain blocks neuronal response to myelin-associated inhibitors in a dominant-negative manner [9]. Neurones from TROY-deficient mice are resistant to the suppressive activity of myelin-associated inhibitors [10]. LINGO-1, also a type I membrane protein, consisting of 12 leucine-rich repeats, an immunoglobulin (Ig)-like domain, a transmembrane domain, and a short cytoplasmic tail containing a canonical epidermal growth factor receptor-like tyrosine phosphorylation site, acts as an adaptor that connects NgR (the ligand-binding component) with $p75^{NTR}$ or TROY (the signal transducing component) by forming the trimolecular complex [13,14]. The expression of LINGO-1 is enriched in the limbic system and the neocortex, identified exclusively in subpopulations of neurones in the rodent CNS [13,15]. Coexpression of NgR, TROY and LINGO-1 confers responsiveness to myelin-associated inhibitors in transfected COS-7 cells [13]. Exposure of cultured rat cerebellar granular (CG) neurones to LINGO-1-Fc fusion protein attenuates Nogo-66, OMgp or MAG-mediated neurite outgrowth inhibition [13]. These observations suggest that the NgR/TROY/LINGO-1 complex plays a key role in inhibiting axonal regeneration in the rodent CNS. However, at present, the precise cellular distribution of TROY and LINGO-1 remains to be characterized in the adult human CNS.

Recently, we demonstrated that Nogo-A expression is enhanced in surviving oligodendrocytes, while NgR is up-regulated in reactive astrocytes and macrophages/microglia in chronic active demyelinating lesions of MS, suggesting a pathological role of Nogo-A/NgR interaction in persistent demyelination and axonal degeneration in MS lesions [16]. In contrast, we found that $p75^{NTR}$ expression is restricted in small regions such as substantia gelatinosa in the spinal cord [16]. The present study by using immunohistochemistry was designed to investigate TROY and LINGO-1 expression in demyelinating lesions of MS.

Materials and methods

Human brain tissues

For immunohistochemistry, 10 micron-thick serial sections were prepared from autopsied brains of five MS patients and 10 non-MS cases. The tissues were fixed with 4% paraformaldehyde or 10% neutral formalin and embedded in paraffin. MS cases included a 29-year-old woman with secondary progressive MS (SPMS) (MS#1), a 40-year-old woman with SPMS (MS#2), a 43-year-old woman with primary progressive MS (PPMS) (MS#3), a 70-year-old woman with SPMS (MS#4) and a 33-year-old man with SPMS (MS#5). Non-MS neurological and psychiatric disease cases included a 47-year-old man with acute cerebral infarction, an 84-year-old man with acute cerebral infarction, a 62-year-old man with old cerebral infarction, a 56-year-old man with old cerebral infarction, a 36-year-old woman with schizophrenia (SCH) and a 61-year-old man with SCH. Neurologically normal control cases included a 79-year-old woman who died of hepatic cancer, a 75-year-old woman who died of breast cancer, a 60-year-old woman who died of external auditory canal cancer and a 74-year-old woman who died of gastric and hepatic cancers.

For Western blot analysis, MS cases included MS#1, #2, #3 and #4. Non-MS cases included a 76-year-old woman with Parkinson's disease (PD#1), a 61-year-old woman with amyotrophic lateral sclerosis (ALS#1), a 74-year-old woman with ALS (ALS#2), a 61-year-old man with ALS (ALS#3), a 66-year-old man with ALS (ALS#4), a 73-year-old man with SCH (SCH#1) and a 77-year-old woman with depression (DEP#1). The *post mortem* interval of the cases ranges from 1.5 h to 10 h prior to freezing the brain tissues. Autopsies were performed either at the National Center Hospital for Mental, Nervous and Muscular Disorders, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan or at the Nishitaga National

Hospital. Sendai. Japan. Written informed consent was obtained form all the cases examined. The present study was approved by the Ethics Committee of NCNP.

Human neural cell lines and cultures

Human astrocytes in culture were established from neuronal progenitor cells isolated from the brain of a human foetus at 18.5-week gestation obtained from BioWhittaker. Walkersville. MD. USA [16]. Human neural cell lines were maintained as described previously [17]. Human microglial cell line HMO6 is provided by Dr Seung U. Kim. Division of Neurology. Department of Medicine. University of British Columbia. Vancouver. BC. Canada [18].

Immunohistochemistry

After deparaffination. tissue sections were heated in 10 mM citrate sodium buffer. pH 6.0 by autoclave at 125°C for 30 s in a temperature-controlled pressure chamber (Dako. Tokyo. Japan). They were treated at room temperature (RT) for 15 min with 3% hydrogen peroxide-containing methanol to block the endogenous peroxidase activity. The tissue sections were then incubated with phosphate-buffered saline (PBS) containing 10% normal rabbit serum or 10% normal goat serum at RT for 15 min to block non-specific staining. The serial sections were incubated in a moist chamber at 4°C overnight with goat anti-TROY antibody (E-19, sc-13711: Santa Cruz Biotechnology. Santa Cruz. CA. USA). rabbit anti-LINGO-1 antibody (#07-678: Upstate. Lake Placid. NY. USA). mouse monoclonal anti-$p75^{NTR}$ antibody (clone ME20.4: Sigma. St Louis. MO. USA) or the antibodies against cell type-specific markers described previously [16]. After washing with PBS. the tissue sections were labelled at RT for 30 min with horseradish peroxidase (HRP)-conjugated secondary antibodies (Nichirei. Tokyo. Japan). followed by incubation with a colourizing solution containing diaminobenzidine tetrahydrochloride (DAB) and a counterstain with haematoxylin. For negative controls. the step of incubation with primary antibodies was omitted. or the tissue sections were incubated with a negative control reagent (Dako) instead of primary antibodies.

In some experiments. tissue sections were initially stained with mouse anti-GFAP monoclonal antibody (GA5: Nichirei). then followed by incubation with alkaline phosphatase-conjugated secondary antibody (Nichirei) and colourized with New Fuchsin substrate. After inactivation of the antibody by autoclaving the sections in 10 mM citrate sodium buffer. pH 6.0. they were relabelled with anti-TROY antibody (sc-13711) or anti-LINGO-1 antibody (#07-678). followed by incubation with peroxidase-conjugated secondary antibody (Nichirei) and colourized with DAB substrate. Immunoreactivity was graded as intense. intermediate. weak or undetectable.

Western blot analysis

To prepare total protein extract. frozen brain tissues prepared from the frontal cerebral cortex or the cerebellar cortex were homogenized in RIPA lysis buffer composed of 50 mM Tris-HCl. pH 7.5. 150 mM NaCl. 1% Nonidet P40. 0.5% sodium deoxycholate. 0.1% SDS. and a cocktail of protease inhibitors (Roche Diagnositics. Tokyo. Japan). followed by centrifugation at 13 400 g for 20 min at RT. The supernatant was collected for separation on a 12% SDS-PAGE gel. The protein concentration was determined by a Bradford assay kit (Bio-Rad. Hercules. CA. USA). After gel electrophoresis. the protein was transferred onto nitrocellulose membranes. and immunolabelled at RT overnight with anti-TROY antibody (sc-13711). anti-LINGO-1 antibody (#07-678). mouse monoclonal anti-NgR antibody (clone 188428: R&D Systems. Minneapolis. MN. USA). anti-$p75^{NTR}$ antibody (ME20.4) or goat anti-heat shock protein HSP60 antibody (N-20. sc-1052: Santa Cruz Biotechnology) for the internal control. Then. the membranes were incubated at RT for 30 min with HRP-conjugated anti-mouse. rabbit or goat IgG (Santa Cruz Biotechnology). The specific reaction was visualized by using a chemiluminescent substrate (Pierce. Rockford. IL. USA). After the antibodies were stripped by incubating the membranes at 50°C for 30 min in stripping buffer composed of 62.5 mM Tris-HCl. pH 6.7. 2% SDS and 100 mM 2-mercaptoethanol. the membranes were processed for relabelling several times with different antibodies.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

DNase-treated total cellular RNA was processed for cDNA synthesis using oligo$(dT)_{12-18}$ primers and SuperScript II reverse transcriptase (Invitrogen. Carlsbad. CA. USA). Then. cDNA was amplified by polymerase chain reaction (PCR) using HotStar Taq DNA polymerase (Qiagen.

Valencia, CA, USA) and a panel of sense and antisense primer sets following: 5′-tcccatgggtggtgacaacatctc-3′ and 5′-gaatgagactggactggaacagcc-3′ for a 159-bp product of TROY; 5′-cctccctacccttctacacacgtt-3′ and 5′-gtgtcggttcg tcggctttcaact-3′ for an 175-bp product of LINGO-1; 5′-agcagccaggtgtgtgtacatacg-3′ and 5′-cgccgaaccctgtaaaca tgatgg-3′ for a 154-bp product of NgR; 5′-gaccacacttcct gtccagagaga-3′ and 5′-atatgacacctgctgtggtgggag-3′ for a 142-bp product of $p75^{NTR}$; and 5′-ccatgttcgtcatgggtgtga acca-3′ and 5′-gccagtagaggcagggatgatgttc-3′ for a 251-bp product of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene as an internal control. The amplification program consisted of an initial denaturing step at 95°C for 15 min, followed by a denaturing step at 94°C for 1 min, an annealing step at 60°C for 40 s and an extension step at 72.9°C for 50 s for 38 cycles, except for G3PDH amplified for 32 cycles. For the positive control, total RNA of the human frontal cerebral cortex (Clontech, Mountain View, CA, USA) was processed in parallel for RT-PCR.

Results

The constitutive expression of TROY and LINGO-1 mRNA in various human neural cell lines and primary cultures

First, the expression of TROY and LINGO-1 mRNA was studied in human neural cell lines and cultures by RT-PCR analysis. High levels of NgR and TROY transcripts were identified in all cell types, including cultured astrocytes, neuronal progenitor cells, NTera2 teratocarcinoma-derived neurones (NTera2N), Y79 retinoblastoma, SK-N-SH neuroblastoma, IMR-32 neuroblastoma, U-373MG astrocytoma, HeLa cervical carcinoma, HepG2 hepatocellular carcinoma and HMO6 microglial cell line, along with in the human cerebral cortex (Figure 1**a**,**b**, lanes 1, 3–12). LINGO-1 and $p75^{NTR}$ transcripts were also detected in various cell lines, although LINGO-1 mRNA levels were fairly low in U-373MG and HeLa, and $p75^{NTR}$ mRNA levels were extremely low in HMO6 cells (Figure 1**c**,**d**, lanes 1, 3–12). The levels of G3PDH mRNA, a housekeeping gene, were almost constant among the cells examined (Figure 1**c**, lanes 1, 3–12), while no products were amplified when total RNA was processed for PCR without inclusion of the reverse transcription step, confirming that a contamination of genomic DNA was excluded (Figure 1**a**–**e**, lane 2).

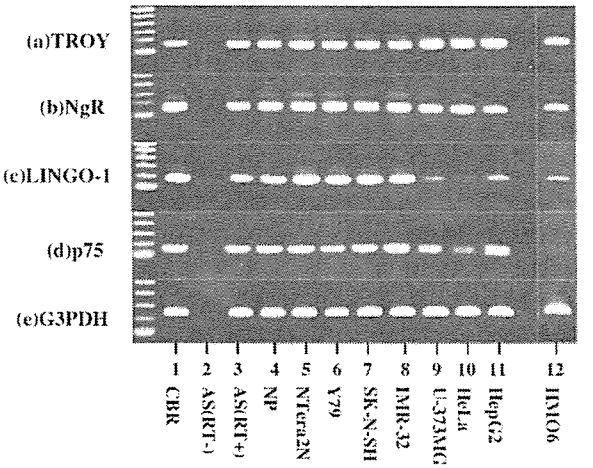

Figure 1. TROY and LINGO-1 mRNA expression in human neural cells. The expression of (a) TROY, (b) NgR, (c) LINGO-1, (d) $p75^{NTR}$ and (e) G3PDH (an internal control) mRNA was studied in human neural cells by RT-PCR analysis. The lanes (1–12) represent: (1) the frontal cerebral cortex (CBR), (2) cultured astrocytes (AS) without inclusion of the reverse transcription step (RT–), (3) cultured astrocytes (AS) with inclusion of the reverse transcription step (RT+), (4) cultured neuronal progenitor (NP) cells, (5) NTera2 teratocarcinoma-derived neurones (NTera2N), (6) Y79 retinoblastoma, (7) SK-N-SH neuroblastoma, (8) IMR-32 neuroblastoma, (9) U-373MG astrocytoma, (10) HeLa cervical carcinoma, (11) HepG2 hepatocellular carcinoma and (12) HMO6 microglial cell line. The DNA size marker (100-bp ladder) is shown on the left.

These results indicate that a panel of human neural cells in culture express both NgR/TROY/LINGO-1 and NgR/$p75^{NTR}$/LINGO-1 complexes.

Neurones, reactive astrocytes and macrophages/microglia but not oligodendrocytes expressed TROY and LINGO-1 in MS and control brains

In the next step, the expression of TROY and LINGO-1 proteins was studied in MS and non-MS brains by immunohistochemistry. A subpopulation of neurones in the cerebral cortex, brainstem and spinal cord expressed constitutively variable intensities of immunoreactivity for TROY or LINGO-1 in both MS and non-MS brains (Figure 2**a**,**b**). An intermediate or weak immunolabelling of TROY or LINGO-1 was identified in a subset of reactive hypertrophic astrocytes in chronic active demyelinating lesions of MS (Figure 2**c**,**d**) and ischaemic lesions of acute cerebral infarction (not shown), although the percentage and intensity of immunopositive cells/total hypertrophic reactive astrocytes was much greater and stronger in

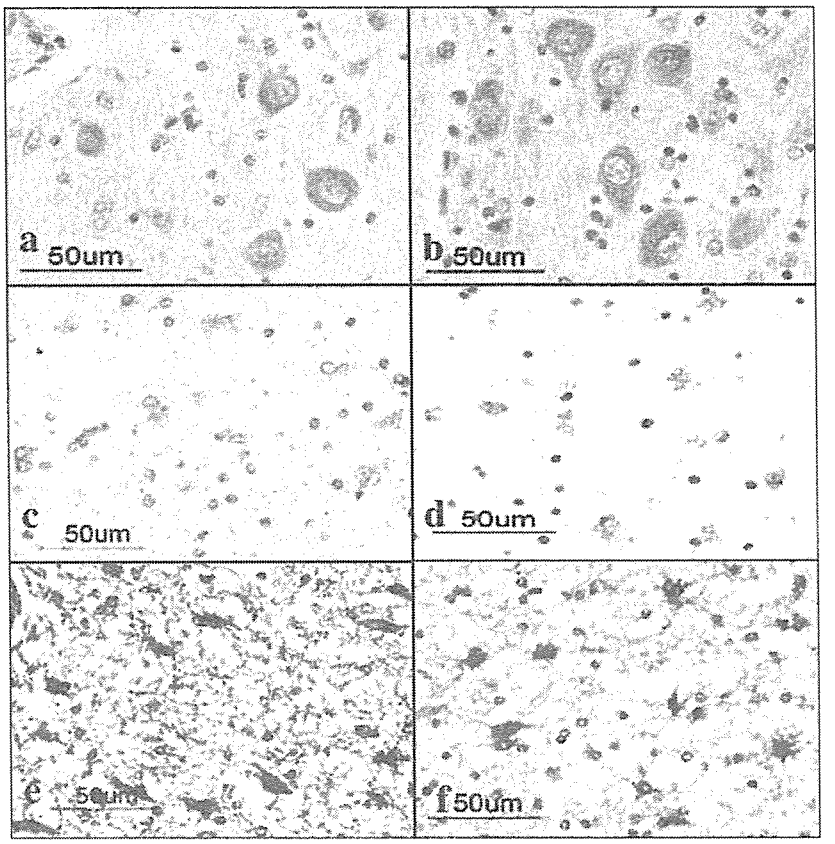

Figure 2. TROY and LINGO-1 immunoreactivities are identified on neurones and reactive astrocytes in multiple sclerosis (MS) brains. The expression of (a,c,e) TROY and (b,d,f) LINGO-1 was studied in MS brains by immunohistochemistry. The panels represent (a) TROY immunoreactivity in the pontine base of MS#1, (b) LINGO-1 immunoreactivity in the pontine base of MS#1, (c) TROY immunoreactivity in chronic active demyelinating lesions in the frontal cerebral cortex of MS#2, (d) LINGO-1 immunoreactivity in chronic active demyelinating lesions in the frontal cerebral cortex of MS#3, (e) TROY (brown) and GFAP (red) double immunolabelling of chronic active demyelinating lesions in the frontal cerebral cortex of MS#2, and (f) LINGO-1 (brown) and GFAP (red) double immunolabelling of chronic active demyelinating lesions in the frontal cerebral cortex of MS#3. Neurones (a,b) and reactive hypertrophic astrocytes (c–f) express TROY and LINGO-1.

TROY (> 80%) than in LINGO-1 (< 10%), suggesting that not all TROY-expressing astrocytes coexpress substantial levels of LINGO-1. Double immunolabelling verified that a population of the cells expressing TROY or LINGO-1 immunoreactivity was comprised of GFAP$^+$ astrocytes (Figure 2e,f). The great majority of macrophages and microglia expressed an intense/intermediate immunoreactivity for TROY and LINGO-1 in MS and non-MS brain lesions (Figure 3a,b). In contrast, both TROY and LINGO-1 was neither detectable in surviving oligodendrocytes remaining in demyelinating lesions nor in oligodendrocytes in the normal appearing white matter of MS (Figure 3c,d) and non-MS brains (not shown). $p75^{NTR}$ expression was restricted in small regions such as substantia gelatinosa in the spinal cord (Figure 3e), as described previously [16].

Up-regulated expression of TROY in MS brains

Finally, the expression of TROY and LINGO-1 proteins was studied in brain tissues of four MS and seven non-MS cases by Western blot analysis. TROY protein levels were much higher in four MS cases MS#1, #2, #3 and #4 (Figure 4a, lanes 1–7), when compared with five non-MS cases PD#1, ALS#1, #2 and #3, and SCH#1 (Figure 4a, lanes 8–14 and 17), or comparable to two non-MS cases ALS#4 and DEP#1 (Figure 4a, lanes 16 and 19), when standardized against the levels of HSP60, an internal standard detected

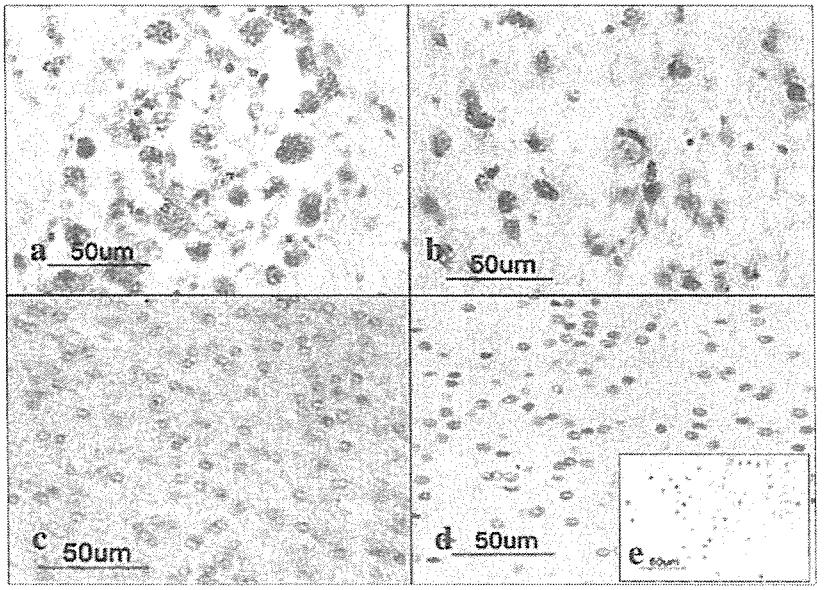

Figure 3. TROY and LINGO-1 immunoreactivities are identified on macrophages/microglia but not on oligodendrocytes in multiple sclerosis (MS) brains. The expression of (a,c) TROY, (b,d) LINGO-1 and (e) $p75^{NTR}$ was studied in MS brains by immunohistochemistry. The panels represent (a) TROY immunoreactivity in chronic active demyelinating lesions in the frontal cerebral cortex of MS#3, (b) LINGO-1 immunoreactivity in chronic active demyelinating lesions in the parietal cerebral cortex of MS#3, (c) TROY immunoreactivity in the normal appearing white matter of the frontal cerebral cortex of MS#1, (d) LINGO-1 immunoreactivity in the normal appearing white matter of the frontal cerebral cortex of MS#1, and an inset, (e) $p75^{NTR}$ immunoreactivity in the substantia gelatinosa of the spinal cord of MS#2. Macrophages/microglia (a,b) but not oligodendrocytes (c,d) express TROY and LINGO-1.

in corresponding blots, which appeared to be almost constant among the samples (Figure 4e, lanes 1–19). In contrast, LINGO-1 protein levels were reduced in all MS cases and the cerebellum of the case of DEP#1, compared with other cases (Figure 4c, lanes 1–7 and 19). The pattern of $p75^{NTR}$ protein expression (Figure 4d, lanes 1–19) was generally similar to that of TROY (Figure 4a, lanes 1–19), except for one case of MS MS#2 that expressed a high level of TROY but a trace of $p75^{NTR}$ (Figure 4a,d, lane 2). In contrast, NgR protein levels varied among the cases and the regions examined, although there existed a trend for higher levels of NgR in the cerebellum *vs.* lower levels in the cerebrum (Figure 4b, lanes 1–19).

Discussion

By immunohistochemistry, the present study for the first time demonstrated that the expression of TROY and LINGO-1 was more widespread than $p75^{NTR}$ in the brains of MS and non-MS cases, identified in subpopulations of reactive astrocytes, macrophages/microglia and neurones but not in oligodendrocytes in chronic active demyelinating lesions of MS and ischaemic lesions of cerebral infarction. The expression of TROY and LINGO-1 in astrocytes and microglia, the findings previously unreported [9–15], was verified by immunohistochemistry of brain tissue sections and RT-PCR analysis of human astrocytes in culture and the microglia cell line HMO6. Non-neuronal distribution of TROY and LINGO-1, both of which constitute pivotal components of the NgR signalling complex [9,10,13], is not so surprising. Previous studies showed that TROY mRNA was detected in glioma and embryonal carcinoma cells [11], and recently, we reported that Nogo-A expression is enhanced in surviving oligodendrocytes, while NgR is up-regulated in reactive astrocytes and macrophages/microglia in chronic active demyelinating lesions of MS, suggesting an active role of NgR signalling in non-neural cell types [16]. Our observations put forth the hypothesis that the ternary complex of NgR/TROY/LINGO-1 expressed on astrocytes, macrophages/microglia and neurones, by interacting with Nogo-A expressed on oligodendrocytes, plays some role in regulating glial–neuronal and glial–glial interactions in active demyelinating lesions of MS.

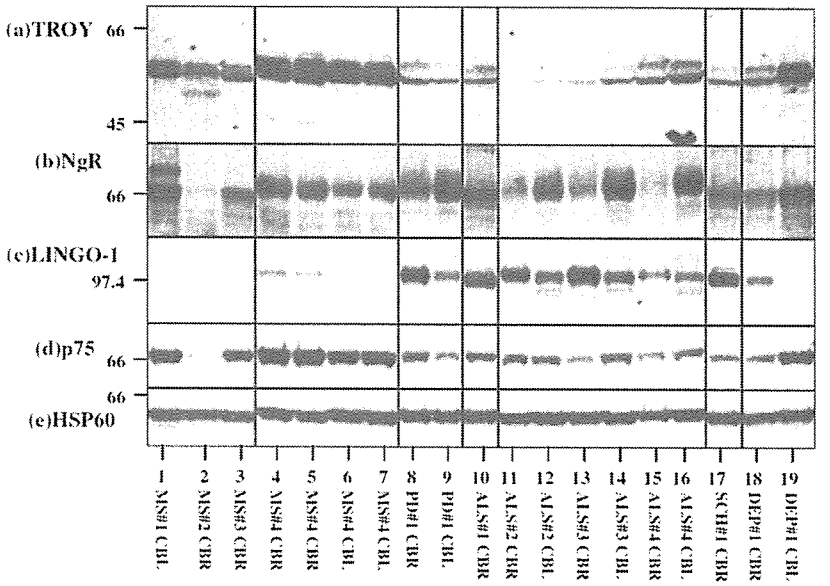

Figure 4. TROY and LINGO-1 protein expression in MS and non-MS brains. The expression of (a) TROY (55 kDa), (b) NgR (67 kDa), (c) LINGO-1 (97 kDa), (d) $p75^{NTR}$ (68 kDa) and (e) HSP60 (60 kDa, an internal control) proteins was studied by Western blot analysis in brain homogenates prepared from either the frontal cerebral cortex (CBR) or the cerebellar cortex (CBL) of four MS and seven non-MS cases. Sixty micrograms of total protein separated on a 12% SDS-PAGE gel was transferred onto nitrocellulose membranes, and processed for relabelling several times with different antibodies. The lanes (1–19) represent (1) MS#1 CBL, (2) MS#2 CBR, (3) MS#3 CBR, (4) MS#4 CBR, (5) a different part of MS#4 CBR, (6) MS#4 CBL, (7) a different part of MS#4 CBL, (8) PD#1 CBR, (9) PD#1 CBL, (10) ALS#1 CBR (11) ALS#2 CBR, (12) ALS#2 CBL, (13) ALS#3 CBR, (14) ALS#3 CBL, (15) ALS#4 CBR, (16) ALS#4 CBL, (17) SCH#1 CBR, (18) DEP#1 CBR and (19) DEP#1 CBL. PD, Parkinson's disease; ALS, amyotrophic lateral sclerosis; SCH, schizophrenia; DEP, depression. The position of molecular weight marker is indicated on the left.

By Western blot analysis, we found that TROY levels were elevated, whereas LINGO-1 levels were reduced in MS brains, although the sample size of the present study was too small, i.e. seven samples of four MS cases *vs.* 12 samples of seven non-MS cases, to obtain definitive conclusions. In contrast, immunohistochemical studies showed that both TROY and LINGO-1 immunoreactivities were enhanced in subpopulations of astrocytes and macrophages/microglia in MS brains. Because a subset of neurones expressed variable levels of TROY and LINGO-1, the discrepancy in the results between Western blot and immunohistochemistry is in part attributable to the varying degree of axonal loss in the lesions examined. Recent studies indicate that LINGO-1 expression is regulated by neuronal activity [19]. The expression of LINGO-1 mRNA is strongly up-regulated, while NgR mRNA levels are decreased in the dentate gyrus of rat brain following treatment with brain-derived neurotrophic factor or kainic acid, suggesting a role of LINGO-1 in activity-dependent neuronal plasticity responses [19]. Reduced neuronal activity causes irreversible axonal damage in demyelinating lesions of MS [20]. Recent studies indicate that LINGO-1 is expressed on rat oligodendrocytes where it negatively regulates differentiation and myelination competence of oligodendrocytes [21]. Expression of dominant-negative LINGO-1, LINGO-1 RNA-mediated interference, or an exposure to soluble LINGO-1-Fc enhances differentiation of rat oligodendrocytes in culture, accompanied by down-regulation of RhoA activity [21]. Furthermore, LINGO-1 knockout mice have greater numbers of myelinated axons [21]. These observations do not apparently agree with our present and previous studies showing that LINGO-1, along with NgR and TROY, was undetectable on oligodendrocytes in the brains of any cases examined [16]. This discrepancy is attributable in part to the differences between the previous study [21] and our own in the species and age, developing rat *vs.* adult human, or to the methods, cell cultures *vs.* immunohistochemistry, or both.

In conclusion, the expression of TROY and LINGO-1 was identified in subpopulations of reactive astrocytes, macrophages/microglia and neurones but not in oligodendrocytes in chronic active demyelinating lesions of MS

and ischaemic lesions of cerebral infarction. These observations suggest that the ternary complex of NgR/TROY/LINGO-1 expressed on astrocytes, macrophages/microglia and neurones, by interacting with Nogo-A expressed on oligodendrocytes, might play a regulatory role in glial–neuronal and glial–glial interactions under demyelinating conditions of MS and other pathological conditions.

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Detection of anti-Nogo receptor autoantibody in the serum of multiple sclerosis and controls

Onoue H, Satoh J-I, Ogawa M, Tabunoki H, Yamamura T. Detection of anti-Nogo receptor autoantibody in the serum of multiple sclerosis and controls.
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Objectives – A myelin-associated neurite outgrowth inhibitor Nogo-A plays a key role in inhibition of axonal regeneration. Axonal damage beginning at the early stage of multiple sclerosis (MS) is responsible for permanent neurological deficits, although its molecular mechanism remains unknown. The aim was to study the prevalence of autoantibodies against Nogo-A and Nogo receptor (NgR) in the serum of MS. *Methods* – The antibodies were identified in the serum of 30 MS patients, 22 patients with non-MS other neurological diseases (OND), and 22 healthy control (HC) subjects by Western blot using recombinant human Nogo-A-specific segment (NAS), the shared segment of Nogo-A and -B (NAB), Nogo-66 (N66), the non-glycosylated form of NgR, the glycosylated NgR (NgR-Fc), and myelin oligodendrocyte glycoprotein (MOG). *Results* – None showed immunoglobulin G (IgG) antibodies against NAS or NAB. In contrast, 30% of MS, 23% of OND and 32% of HC subjects exhibited anti-N66 IgG, while 27% of MS, 27% of OND and 18% of HC showed anti-MOG IgG. None of HC but 33% of MS and 14% of OND showed anti-non-glycosylated NgR IgG. Furthermore, 60% of MS, 18% of OND and 14% of HC showed anti-NgR-Fc IgG. *Conclusions* – Because IgG autoantibodies against N66, NgR and MOG are often detected in the serum of MS and controls, they do not serve as an MS-specific marker.

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Key words: autoantibodies; multiple sclerosis; Nogo-A; Nogo receptor; Western blot

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Introduction

Axonal damage beginning at the early stage of multiple sclerosis (MS) is responsible for permanent neurological deficits and progression of clinical disability (1, 2). Although the underlying molecular mechanism remains unknown, the degree of inflammatory demyelination correlates with the extent of axonal damage, suggesting an involvement of proinflammatory mediators in development of axonal degeneration in MS (3). The alternative possibility could be proposed that axonal regeneration is severely impaired in MS lesions, because an extensive accumulation of neurite outgrowth inhibitors and glial scar provides a non-permissive environment for axonal regrowth (4).

Nogo constitutes a family of myelin-associated inhibitors of axonal regeneration (5, 6). The Nogo gene encodes three distinct isoforms named A, B and C, derived by alternative splicing and promoter usage, all of which share a C-terminal 66 amino acid segment named Nogo-66 located between the two transmembrane domains. Nogo-A, the longest isoform, is the major neurite outgrowth inhibitor expressed on oligodendrocytes and myelin sheath and a subpopulation of neurons in the central nervous system (CNS) (7, 8). Nogo-A has at least two discrete regions with neuronal growth-inhibitory activities. One is located in the Nogo-A-specific region that restricts neurite outgrowth, while another is Nogo-66 that induces growth cone collapse, both of which assume different membrane topologies depending on cell types (9). Nogo-66

binds to the Nogo receptor (Nogo-66 receptor-1; NgR), a glycosylphosphatidylinositol-anchored membrane protein (10). In contrast to Nogo-A, NgR is not identified on oligodendrocytes but expressed in a subpopulation of neurons and their axons, including cerebral cortical pyramidal neurons and cerebellar Purkinje cells (10, 11). Signal transduction mediated by NgR depends on its association with a coreceptor $p75^{NTR}$ or TROY in combination with an adaptor molecule LINGO-1 (12–14). Not only Nogo-66 but also myelin-associated glycoprotein and oligodendrocyte-myelin glycoprotein bind to NgR and transduce neurite growth-inhibitory signals via $p75^{NTR}$ by activating RhoA and inhibiting Racl (15). *In vivo* blockade of interaction between NgR and its ligands, by neutralizing anti-Nogo-A antibodies, NgR antagonistic peptides, or soluble truncated NgR, induces extensive axonal regeneration and enhances functional recovery after injury in the CNS (16–18). Furthermore, NgR-deficient mice exhibit an enhanced axonal plasticity after ischemic stroke, accompanied by improved functional recovery (19).

Increasing evidence indicates that autoantibodies against CNS antigens play a key role in the immunopathogenesis of MS. The detection of anti-myelin oligodendrocyte glycoprotein (MOG) antibody in the patients with clinically isolated syndrome (CIS) predicts the early conversion of CIS to clinically definite MS (20). The detection of immunoglobulin G (IgG) autoantibody against aquaporin-4 discriminates the patients with neuromyelitis optica from those with the conventional MS (21). Recently, autoantibodies against the Nogo-A segment spanning amino acid residues 1–979 are identified in the serum of relapsing–remitting MS (RRMS) patients, although they show no significant correlation to disease course, duration, relapse rate and disability of the patients (22). More recently, we found that Nogo-A expression is enhanced in surviving oligodendrocytes, while NgR is upregulated in reactive astrocytes at the edge of chronic demyelinating lesions of MS, suggesting that Nogo-A/NgR mediates glial–glial interaction under demyelinating environments (23). Inflammatory demyelination and axonal damage are less severe in Nogo-A-deficient mice affected with experimental autoimmune encephalomyelitis, an animal model of MS (24). These observations propose a possible scenario that the Nogo-A/NgR interaction restricts axonal regeneration in MS brain.

The present study is designed to investigate the prevalence of autoantibodies against a panel of Nogo and NgR fragments in the serum of MS and controls by Western blot analysis, and to determine the possible association of these antibodies to clinical profiles of MS.

Patients and methods

Study population

The serum samples were obtained from randomly selected 30 MS patients, 22 patients with other neurological diseases (OND) who visited the National Center Hospital for Mental, Nervous and Muscular Disorders, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan in 2004, and 22 healthy control (HC) subjects. Written informed consent was obtained from all the subjects. MS was diagnosed following the established criteria (25) by qualified neurologists of the hospital. The MS population was composed of 10 men and 20 women showing the mean age of 41.5 ± 12.2 years, the mean disease duration of 7.1 ± 6.3 years, and the mean Expanded Disability Status Scale (EDSS) score of 3.6 ± 2.0. The MS group included 17 patients with RRMS during remission, nine with RRMS in acute relapse, three with secondary progressive MS (SPMS) and one with primary progressive MS (PPMS). Among all MS patients, nine were under IFNβ treatment, 11 were treated with corticosteroids, and two with azathioprine at the time of blood sampling. The patients received no other immunosuppressive or immunomodulatory drugs. The OND population included 13 men and nine women with the mean age of 57.7 ± 13.8 years, composed of six patients with spinocerebellar degeneration, five with Parkinson's disease, three with myopathy, two with neuropathy, one with myasthenia gravis, Fisher syndrome, adrenoleukodystrophy, spastic paraplegia, epilepsy, or cerebral infarction. The HC population was composed of nine men and 13 women presenting with the mean age of 41.7 ± 12.4 years. After sampling, the serum samples were stored immediately at −30°C before use.

Recombinant human Nogo and NgR fragments

The human Nogo-A gene encoding the Nogo-A-specific segment (NAS; amino acids 186–1004), the segment shared between Nogo-A and Nogo-B (NAB; amino acids 1–185), and the Nogo-66 segment (N66; amino acids 1053–1118), the human NgR gene encoding the full-length NgR following a cleavage of the N-terminal signal peptide (amino acids 27–473), and the human MOG gene encoding the full-length MOG

Table 1 Primers utilized for PCR-based cloning

Genes	Proteins (symbol, amino acid residues)	GenBank accession no.	Sense primers	Antisense primers
RTN4	Nogo-A-specific segment (NAS, 186–1044)	NM_020532	5′-gatgagacccttttgctcttcct-3′	5′-tcatgaagttttactcagctctgctga-3′
RTN4	Nogo-A/B-shared segment (NAB, 1–185)	NM_020532	5′-atggaagaactggaccagtctcct-3′	5′-tcacactgagcccgaggagcccct-3′
RTN4	Nogo-66 segment (N66, 1053–1118)	NM_020532	5′-agctttaggatatacaagggtgtg-3′	5′-tcaagaatcaactaaatcatcaactaa-3′
RTN4R	Nogo receptor without an N-terminal signal peptide (NgR, 27–473)	NM_023004	5′-tgcccaggtgcctgcgtatgctacaat-3′	5′-tcagcagggcccaagcactgtccacag-3′
MOG	Myelin oligodendrocyte glycoprotein without an N-terminal signal peptide (MOG, 30–247)	NM_206809	5′-gggcagttcagagtgataggaccaaga-3′	5′-tcagaagggatttcgtagctcttcaagg-3′

The PCR product was cloned into a prokaryotic expression vector pTrcHis-TOPO to produce a fusion protein with an N-terminal Xpress tag in *E. coli*.

following a cleavage of the N-terminal signal peptide (amino acids 30–247) were amplified by PCR using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA) from cDNA of NTera2-derived human neurons (26) or human brain using a panel of sense and antisense primer sets listed in Table 1. The PCR product was cloned into a prokaryotic expression vector pTrcHis-TOPO (Invitrogen, Carlsbad, CA, USA). After transformation of the vector into *Escherichia coli*, the expression of recombinant proteins was induced by exposing the culture of *E. coli* to isopropyl β-thiogalactoside. All recombinant proteins having an N-terminal Xpress tag for detection were purified from the *E. coli* lysate by isolating the specific bands immunoreactive with monoclonal anti-Xpress antibody (Invitrogen) separated on an 8%, 12% or 15% SDS-PAGE gel. The recombinant proteins were eluted from the gels, precipitated by cold acetone, and dissolved in distilled water containing 0.1% SDS. The protein concentration was determined by a Bradford assay kit (BioRad, Hercules, CA, USA). For control, the pTrcHis-TOPO vector containing a *lacZ* gene fragment (Invitrogen) was transformed into *E. coli* in parallel. Recombinant human NgR (Met1-Ser447)-IgG Fc (Pro100-Lys330) fusion protein (95 kDa), a glycosylated form expressed in a mouse myeloma cell line, was obtained from R&D Systems (Minneapolis, MN, USA).

Western blot analysis

Six different recombinant proteins, including NAS, NgR, LacZ, NAB, MOG and N66, were separated on a single 12% SDS-PAGE gel. After gel electrophoresis, they were transferred onto a nitrocellulose membrane. This membrane is designated the miniarray. Then, the array was incubated at room temperature (RT) overnight with the serum diluted at a concentration of 1:1000 in phosphate-buffered saline containing 5% skim milk. It was followed by incubation at RT for 60 min with horseradish peroxidase (HRP)-conjugated anti-human IgG (160 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with an HRP-conjugated antibody specific for human IgM $Fc_{5\mu}$ fragment (8 ng/ml; Jackson ImmunoResearch, West Grove, PA, USA). To identify autoantibodies against NgR-Fc, the serum was diluted at a concentration of 1:15,000, and either an HRP-conjugated antibody specific for human IgG $F(ab')_2$ fragment (20 ng/ml; Jackson ImmunoResearch) or a HRP-conjugated antibody against human IgM $Fc_{5\mu}$ fragment (120 ng/ml; Jackson ImmunoResearch) was utilized as a secondary antibody. The specific reaction was visualized at 15 min after incubating the array for 5 min with a chemiluminescent substrate (Pierce, Rockford, IL, USA). The optimal concentration of the serum, secondary antibodies, and incubation time prior to development was determined to obtain the maximum signal/background intensity by preliminary experiments that include varying combinations of serially diluted sera and secondary antibodies, and incubation periods. The signal intensity was graded as (−) none, (+) weak, (++) strong, and (+++) extremely strong.

Statistical analysis

The statistical difference in clinical profiles between the serum autoantibody-positive and negative groups of MS patients was evaluated by Mann–Whitney *U*-test. A P value of <0.05 was considered statistically significant.

Results

Miniarray of Nogo and NgR fragments

A panel of recombinant proteins, such as NAS, NgR, LacZ, NAB, MOG and N66, all of which have an N-terminal Xpress tag, were separated on a single 12% SDS-PAGE gel and transblotted onto