25), protein kinase, DNA-activated, catalytic subunit (PRKDC; No. 26), Ras association domain family 1 (RASSF1; No. 27), DAXX (No. 29), and epidermal growth factor (EGF; No. 29). The coordinate upregulation of proapoptotic and antiapoptotic genes such as RIPK2, MAD, and SODD suggests that the gene expression pattern in non-T cells in MS also represents a counterbalance between inducing and suppressing apoptosis.

Upregulated genes in MS were expressed in cultured PBMC in an activation-dependent manner

To identify the stimuli affecting the expression of apoptosis signaling-related genes, PBMCs were in vitro exposed to PMA plus IOM, anti-CD3 mAb, or IFNγ. PBMC treated with PMA plus IOM or anti-CD3 mAb showed marked upregulation of CD69, a marker for early activation of lymphocytes, while those exposed to IFNγ exhibited the highest level of IFN-induced 15-kDa protein (ISG15) (Figs. 3a and c, lanes 2–4). IFN regulatory factor 1 (IRF-1) was induced equally by all these stimuli (Fig. 3b, lanes 2–4). These results indicated that PBMC in vitro responded efficiently to PMA plus IOM, anti-CD3 mAb, and IFNγ. PBMC exposed to PMA plus IOM showed the highest level of expression of NR4A2, ICAM1, RIPK2, and CXCL2 (Figs. 3e, g, i, and l, lane 2) while those treated with anti-CD3 mAb exhibited more marked upregulation of CDC42, SODD, and TOP2A (Figs. 3h, m, and n, lane 3). In contrast, IL1R2 and MAD levels were reduced by exposure to PMA plus IOM (Figs. 3j and k, lane 2). PBMC treated with IFNγ did not show substantial upregulation of NR4A2, TCF8, IL1R2, MAD, CXCL2, or TOP2A (Figs. 3e, f, j, k, l, and n, lane 4). The expression of CYP1A2 mRNA was not detected in PBMC incubated under any culture conditions examined (not shown). These results suggest that the genes upregulated in MS were mostly expressed at significant levels in PBMC in vitro in an activation- and stimulation-dependent manner.

Discussion

In the present study, we have investigated the comprehensive gene expression profile of T cells and non-T cells of 72 MS patients and 22 CN subjects. Among 1258 genes on a cDNA microarray, 173 genes in T cells and 50 genes in non-T cells were expressed differentially between MS and CN groups. The great majority of the top 30 significant genes were categorized into apoptosis signaling-related genes of both proapoptotic and antiapoptotic classes. Northern blot analysis showed that most significant genes on microarray were actually expressed in PBMC in vitro at substantial levels in an activation-dependent manner. Our observations suggest that the gene expression pattern in PBMC of MS represents a counterbalance between promoting and preventing apoptosis of lymphocytes, which are ceaselessly exposed to exogenous and endogenous apoptosis-inducing stimuli and stresses (Fig. 4). Because the elimination of pathogenic autoreactive T cells is a pivotal step in the homeostasis of the immune system, dysregulation of apoptosis contributes to the autoimmune pathogenesis of MS. Therefore, it is worthy to note how the genes

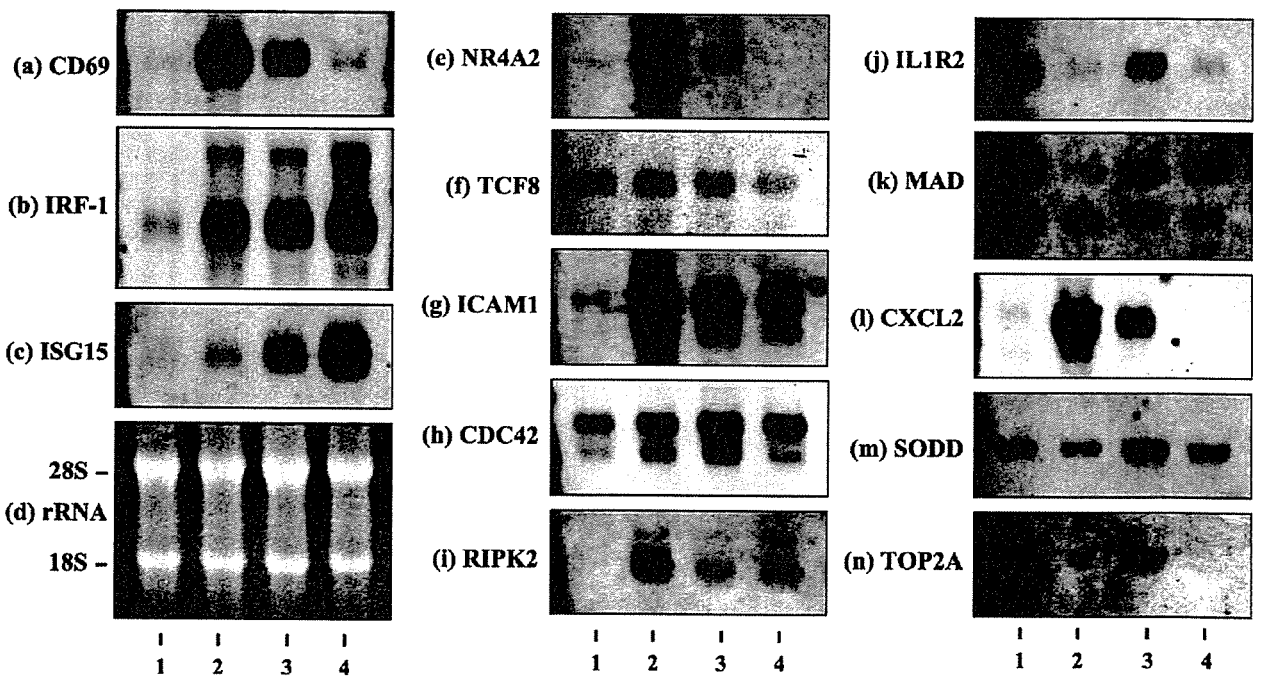

Fig. 3. The genes upregulated in MS were expressed in cultured PBMC in an activation-dependent manner. Unfractionated PBMCs of a healthy subject were incubated for 6 h in medium without (lane 1) or with inclusion of 25 ng/ml PMA and 1 μg/ml IOM (lane 2), or for 24 h in the plate coated with 1 μg/ml anti-CD3 mAb (lane 3) or in the medium containing 100 ng/ml IFNγ (lane 4). They were then processed for RNA preparation. Three micrograms of total RNA was separated on a 1.5% agarose–6% formaldehyde gel and transferred onto a nylon membrane. The membranes were hybridized with the DIG-labeled DNA probe specific for CD69 (panel a), IFN regulatory factor 1 (IRF-1; panel b), IFN-induced 15-kDa protein (ISG15; panel c), NR4A2 (panel e), transcription factor (TCF8) (panel f), ICAM1 (panel g), CDC42 (panel h), RIPK2 (panel i), IL-1 receptor type II (IL1R2) (panel j), Max dimerization protein (MAD) (panel k), chemokine, CXC motif, ligand 2 (CXCL2) (panel l), silencer of death domains (SODD) (panel m), and topoisomerase 2 alpha (TOP2A) (panel n). The ethidium bromide staining of the representative gel is shown in the panel d.

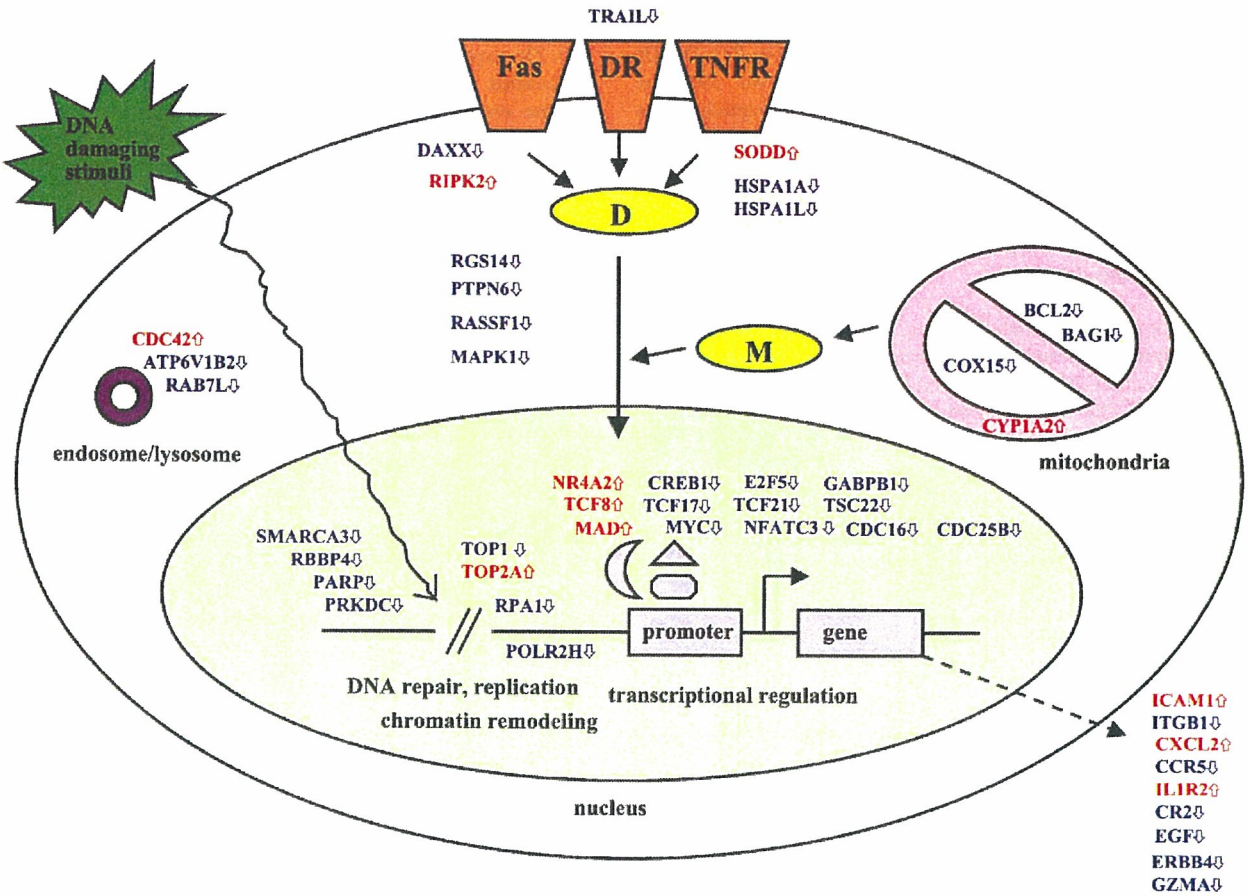

Fig. 4. Aberrant expression of apoptosis signaling-related genes in MS lymphocytes. More than 80% of the top 30 differentially expressed genes between MS and CN groups were categorized into apoptosis signaling-related genes of both proapoptotic and antiapoptotic classes, whose expression was either upregulated (⇑, red) or downregulated (⇓, blue) in MS. The expression of a subset of genes involved in DNA repair, replication, and chromatin remodeling was also dysregulated in MS. The figure represents an integrated view of the results derived from both T cell and non-T cell fractions. Abbreviations: DR, death receptor; TNFR, TNF receptor; D, the DR/Fas/TNFR-mediated apoptosis-signaling pathway; M, the mitochondria-mediated apoptosis-signaling pathway. See Tables 2 and 3 for description of the gene symbols.

identified by microarray analysis play a role in lymphocyte apoptosis.

The genes involved in thymic T cell development

Microarray analysis identified an aberrant expression in MS of important regulators of T cell development. NR4A2, the most significantly upregulated gene in MS T cells, encodes an orphan member of the steroid–thyroid hormone receptor superfamily designated Nurr1. Importantly, Nurr1 is induced in human T cells during apoptosis (Okabe et al., 1995). The members of this family positively regulate clonal deletion of self-reactive T cells in the thymus (Zhou et al., 1996). TCF8 upregulated in MS T cells encodes a transcriptional repressor for the IL-2 gene (Williams et al., 1991). Thymocyte development is impaired in mice expressing the mutant TCF8 (Higashi et al., 1997). CREB1 downregulated in MS T cells is a leucine zipper-containing transcription factor. A homodimer of CREB1, phosphorylated by protein kinase A (PKA), binds to the cAMP-responsive element (CRE) located in the promoter of the genes pivotal for T cell function (Barton et al., 1996). Thymocytes and T cells of transgenic mice expressing a dominant-negative mutant CREB show a profound proliferative defect caused by apoptotic death following activation (Barton et al., 1996). TRAIL downregulated in MS T cells is a type II membrane protein of the TNF family that induces apoptosis preferentially in transformed cells via the death receptors DR4 and DR5. A previous study by using RT-PCR analysis showed that TRAIL mRNA levels are elevated in PBMC of MS (Huang et al., 2000). The discrepancy between this study and our observations might be derived from differences in the study populations and the methods employed. Supporting our findings, a recent study showed that serum soluble TRAIL levels are reduced in RRMS (Wandinger et al., 2003). TRAIL-deficient mice presenting with a severe defect in thymocyte apoptosis are hypersensitive to induction of autoimmune diseases (Lamhamedi-Cherradi et al., 2003). NFATC3 downregulated in MS non-T cells is expressed chiefly in double-positive thymocytes during development. Development of CD4 and CD8 single positive thymocytes and peripheral T cells is impaired in mice lacking NFATC3, accompanied by increased apoptosis of double-positive thymocytes (Oukka et al., 1998). It remains unknown whether these observations reflect an aberrant regulation of thymic T cell development in MS. However, we assume that these alterations appreciably affect the homeostasis of peripheral T cells in MS.

The genes involved in oxidative stress in mitochondria

Microarray analysis identified an aberrant expression in MS of key regulators of oxidative stress. CYP1A2 upregulated in MS T cells encodes a mitochondrial enzyme of the cytochrome *P*450 superfamily that regulates the metabolism of drugs, toxic chemicals, and carcinogens. It plays a role in oxidative stress-induced apoptosis (Nebert et al., 2000). It is worthy to note that cigarette smoking that increases the amount of CYP1A2 in human liver microsomes (Nakajima et al., 1999) is one of risk factors for development of MS (Riise et al., 2003). COX15 downregulated in T and non-T cells of MS encodes a mitochondrial inner membrane protein that promotes the biogenesis of COX. COX is the terminal component of the mitochondrial respiratory chain that provides an antioxidant defense in mitochondria. GABPB1 upregulated in MS T cells regulates transcription of the COX gene. Persistent inhibition of COX by nitric oxide induces the formation of peroxynitrite, a potent inducer of apoptotic cell death (Moncada and Erusalimsky, 2002). These observations raise the possibility that MS lymphocytes are continuously exposed to oxidative stress, although the present study has no detailed information on the history of smoking habits, alcohol consumption, and the use of over-the-counter (OTC) medications in MS and CN groups, all of which are potentially involved in oxidative stress-mediated gene regulation.

The genes involved in lymphocyte recruitment in the CNS

Microarray analysis identified an aberrant expression in MS of several regulators of lymphocyte recruitment. ICAM-1, the most significantly upregulated gene in MS non-T cells, is a ligand for lymphocyte function-associated antigens LFA-1 and Mac-1. ICAM-1, expressed on activated endothelial cells, T cells, B cells, and monocytes, regulates lymphocyte trafficking into the CNS. Importantly, a costimulatory signal through ICAM-1 protects T cells from apoptosis by upregulating the expression of BCL2 (Kohlmeier et al., 2003). A previous study showed that serum-soluble ICAM-1 levels are elevated in active MS, being consistent with our observations (Khoury et al., 2000). ITGB1 downregulated in MS T cells encodes a common beta chain of the very late activation (VLA) protein family. The interaction of VLA4 on T cells with VCAM-1 on endothelial cells is a pivotal step for the recruitment of activated T cells into the CNS through the blood–brain barrier in MS (Calabresi et al., 1997). Again, the activation of ITGB1 inhibits apoptosis of $CD4^+$ T cells (Stallmach et al., 2001). CCR5 downregulated in MS T cells is a receptor specific for RANTES, MIP1α, MIP1β, MCP2, and macrophage-tropic HIV virus. It is expressed predominantly in polarized Th1 T cells (Bonecchi et al., 1998). The interaction of CCR5 with a HIV Env protein upregulates FasL expression, leading to a Fas-dependent apoptotic death of HIV-uninfected $CD4^+$ T cells (Algeciras-Schimnich et al., 2002). A previous study showed that the number of $CCR5^+$ T cells producing high levels of IFNγ is increased in progressive MS but not in RRMS, suggesting that they play a role in the conversion of two distinct clinical phases of MS (Balashov et al., 1999). CXCL2 downregulated in MS non-T cells is a member of the CXC subfamily of chemokines produced chiefly by macrophages and monocytes. It acts as a chemotactic factor for polymophonuclear leukocytes and natural killer (NK) T cells by binding to CXCR2, the receptor shared with IL-8. Macrophages, when they phagocytize apoptotic cells, produce a large amount of CXCL2 (Kurosaka et al., 2003).

Apoptosis-regulatory genes whose involvement is unpredicted in MS

Microarray analysis highlighted several apoptosis regulators whose role in MS has been previously unreported. RIPK2 upregulated in MS non-T cells is a RIP-related protein kinase containing an N-terminal kinase domain and a C-terminal caspase activation and recruitment domain (CARD), a homophilic interaction motif that mediates the recruitment of caspases (Inohara et al., 1998). RIPK2 interacts with CLARP, a caspase-like molecule known to bind to Fas-associated protein with death domain (FADD) and caspase-8. Overexpression of RIPK2 potentiates Fas-mediated apoptosis by activation of nuclear factor-κB (NF-κB), Jun NH_2-terminal kinase (JNK), and caspase-8 (Inohara et al., 1998). Importantly, Th1 differentiation and cytokine production are severely impaired in RIPK2-deficient mice (Kobayashi et al., 2002). DAXX downregulated in both T and non-T cells of MS, by binding to the death domain (DD) of Fas, enhances Fas-induced apoptosis following activation of apoptosis signal-regulating kinase 1 (ASK1) and the JNK pathway (Yang et al., 1997). MAD upregulated in MS non-T cells mediates antiapoptotic activities by forming a heterodimer with MAX, which acts as a transcriptional repressor of MYC-MAX target genes (Zhou and Hurlin, 2001), whereas MYC downregulated in MS T cells enhances cell susceptibility to TNF-mediated apoptosis following inhibition of NF-κB activation (You et al., 2002). SODD upregulated in MS non-T cells, by binding to the DD of TNFR1 and death receptor DR3, blocks the post-receptor signal transduction (Jiang et al., 1999). SODD has a BAG domain that targets the heat shock protein HSP70 at the cytoplasmic domain of TNFR1 (Tschopp et al., 1999). The HSP70 family protects cells against apoptosis by sequestering apoptotic protease activating factor-1 (Apaf-1) (Beere and Green, 2001). HSP70 upregulated in MS brain lesions facilitates processing of myelin basic protein by antigen-presenting cells (Cwiklinska et al., 2003). However, the expression of HSPA1A and HSPA1L, two HSP70 members, was reduced in T and non-T cells of MS.

BCL2 downregulated in MS non-T cells is an integral mitochondrial inner membrane protein that blocks the apoptotic cell death. BAG1 downregulated in MS T cells binds to BCL2 and enhances the antiapoptotic activity of BCL2 (Takayama et al., 1995). CR2 downregulated in MS non-T cells is the membrane receptor termed CD21 specific for the C3d fragment of activated C3. CR2 expressed mainly on B cells and follicular dendritic cells is upregulated by NF-κB activation (Fearon and Carroll, 2000). The CD21, CD19, and CD81 complex enhances signaling through B cell antigen receptor, associated with upregulation of BCL2 expression (Roberts and Snow, 1999).

The genes involved in DNA repair, replication, and chromatin remodeling

Microarray analysis identified an aberrant expression in MS of a battery of regulators of DNA repair, replication, and chromatin remodeling. Most of them were downregulated in MS. DNA topoisomerase (TOP) is a nuclear enzyme that alters the topologic states of DNA. TOP1 downregulated in MS T cells cuts and rejoins a single-stranded DNA, while TOP2A upregulated in MS non-T cells catalyzes a double-stranded DNA and mediates the caspase-independent excision of DNA loop domains during apoptosis

(Solovyan et al., 2002). SMARCA3 downregulated in T and non-T cells of MS belongs to a member of the SWI/SNF family of chromatin remodeling enzymes with DNA helicase activity (Sheridan et al., 1995). The SWI/SNF family protein, by interacting with MYC, facilitates transcriptional activation of several apoptosis-regulatory genes (Klochendler-Yeivin et al., 2002). RBBP4 downregulated in MS non-T cells is a component of the retinoblastoma (Rb) protein-associated histone deacetylase complex that represses transcription of E2F-responsive proapoptotic genes (Nicolas et al., 2000). E2F5 downregulated in MS non-T cells acts as a Smad cofactor that transduces the TGFβ receptor signal to repress transcription of MYC (Chen et al., 2002).

PARP downregulated in MS T cells is a chromatin-associated enzyme that modifies nuclear proteins by polyADP-ribosylation, thereby involved in the maintenance of genomic stability. PARP is cleaved by caspase-3 at the onset of apoptosis (Nicholson et al., 1995). RPA1 downregulated in MS non-T cells is a single-stranded DNA-binding protein associated with a large RNA polymerase II (POLR2) complex, which regulates gene transcription, DNA replication, and repair. POLR2H encoding the H subunit of POLR2 was downregulated in non-T cells of MS. Following DNA damage, RPA1 is phosphorylated by DNA-dependent protein kinase (DNA-PK), a nuclear serine/threonine protein kinase activated upon binding to double-stranded DNA brakes (Wold, 1997). DNA-PK plays a crucial role in V(D)J recombination, maintenance of chromatin and telomere structure, regulation of transcription, and apoptosis (Smith and Jackson, 1999). A nonsense mutation in the PRKDC gene encoding the catalytic subunit of DNA-PK causes the phenotype of severe combined immunodeficiency (SCID) mice that are devoid of mature T and B lymphocytes. PRKDC was also downregulated in non-T cells of MS. GZMA downregulated in MS non-T cells encodes a cytotoxic T lymphocyte- and NK cell-specific serine protease that mediates caspase-independent apoptosis of target cells by creating single-stranded DNA breaks, followed by cleavage of apurinic endonuclease-1, the rate-limiting enzyme of DNA base excision repair (Fan et al., 2003).

Transcription factors and signal transducers involved in regulation of apoptosis

Finally, microarray analysis identified an aberrant expression in MS of various transcription factors and signal transducers involved in regulation of apoptosis. MAPK1 downregulated in MS T cells is a member of the MAP kinase family serine/threonine kinases that play a role in protection of cells from apoptosis (Allan et al., 2003). RGS14 downregulated in T and non-T cells of MS, a member of GTPase-activating protein family, attenuates IL-8 receptor-mediated MAPK activation (Cho et al., 2000). TCF17 downregulated in T and non-T cells of MS is a zinc finger-containing transcriptional repressor that induces nucleolar fragmentation in overexpressing cells (Huang et al., 1999). TCF21 downregulated in MS T cells encodes a member of the basic helix-loop-helix family of transcription factors. TCF21-deficient mice show extensive apoptosis of splenic precursor cells during development (Lu et al., 2000). TSC22 downregulated in MS T cells is a TGFβ-inducible transcription factor. Overexpression of TSC22 induces apoptotic death of gastric cancer cells following activation of caspase-3 (Ohta et al., 1997). RASSF1 downregulated in MS non-T cells is a tumor suppressor gene with a Ras association domain. Overexpression of RASSF1 induces apoptotic death of HEK293 cells, while it is frequently downregulated in lung and ovarian tumor cells (Vos et al., 2000).

CDC42 upregulated in MS non-T cells is a central member of the Rho subfamily of small GTPases. CDC42 regulates cell morphology, migration, endocytosis, cell cycle progression, and apoptosis (Aspenström, 1999). It serves as a substrate for caspases in the Fas-signaling pathway (Tu and Cerione, 2001). Rab7L1 downregulated in non-T cells of MS belongs to a family of Ras-related small GTP-binding proteins that regulate vesicular transport in specific intracellular compartments. Rab7 located in the late endosome plays a role in the ingestion of apoptotic cells by phagocytes. ATP6V1B2 downregulated in MS T cells encodes a subunit of vacuolar H^+-ATPase (V-ATPase) that mediates acidification of endosomal and lysosomal compartments. Concanamycin A, a specific V-ATPase inhibitor, induces apoptosis of B cells (Akifusa et al., 1998). CDC25B downregulated in MS non-T cells regulates G_2-M progression in the cell cycle following activation of CDC2 protein kinase by dephosphorylation. Overexpression of CDC25B enhances apoptosis in cancer cells (Miyata et al., 2001). CDC16 downregulated in MS T cells is a component of the anaphase-promoting complex, a ubiquitin ligase responsible for cyclin A and B degradation, which is inactivated during Fas-induced apoptosis in Jurkat cells (Zhou et al., 1998).

PTPN6 downregulated in MS T cells encodes a cytoplasmic protein-tyrosine phosphatase named SHP-1. It inactivates several receptor and non-receptor tyrosine kinases by dephosphorylation, and plays a role in induction of apoptosis upstream BCL2 (Thangaraju et al., 1999). AKAP11 downregulated in MS non-T cells belongs to a family of scaffolding molecules that regulate the spatial and temporal location of PKA. AKAP11, by forming a complex with the regulatory subunit of PKA and type 1 protein phosphatase, inhibits glycogen synthase kinase-3β, a key enzyme involved in regulation of apoptosis (Tanji et al., 2002). EGF downregulated in MS non-T cells induces apoptosis of A431 epidermoid carcinoma cells following upregulation of caspase-1 in a STAT-dependent manner (Chin et al., 1997). ERBB4 downregulated in MS T cells encodes a member of EGF receptor-related receptor tyrosine kinase family that interacts with neuregulins. Neuregulin signaling activates Akt in oligodendrocytes, a serine/threonine kinase with an antiapoptotic activity (Flores et al., 2000).

Thus, microarray analysis identified an aberrant expression of a wide range of apoptosis and DNA damage-regulatory genes in T and non-T cells of MS. This may represent a counterbalance between promoting and preventing apoptosis of lymphocytes in MS.

Confounding factors that might affect the gene expression levels

Recent studies suggested that gene expression patterns in peripheral blood lymphocytes show interindividual and intra-individual variation (Whitney et al., 2003). Some features of this variation are associated with differences in the cellular composition of the blood sample, with gender, age, and the time of day at which the sample was taken (Whitney et al., 2003). Our study included 72 MS patients and 22 age- and sex-matched healthy CN subjects, and paid special attention to sample handling and processing. All the blood samples were taken in the morning, and PBMCs were isolated within 6 h after sampling. Immediately, they were separated into a $CD3^+$ T cell fraction and a $CD3^-$ non-T cell fraction to prepare total RNA. The purity of each fraction verified by flow cytometric analysis usually exceeded 90–95%. However,

subclinical infection at the time of blood sampling accounting for the variability in gene expression levels (Whitney et al., 2003) could not be excluded in the present study.

Other important factors that potentially affect the gene expression profile in human peripheral blood lymphocytes on microarray include the recent use of OTC medications, smoking, alcohol intake, and the menstrual condition. Aspirin, one of nonsteroid anti-inflammatory drugs (NSAIDs), affects the expression pattern of several genes related to cell growth inhibition in human colon cancer cells (Iizaka et al., 2002). Nicotin, a major constituent of cigarette smoke, alters the expression of genes involved in signal transduction and transcriptional regulation in human coronary artery endothelial cells (Zhang et al., 2001). Microarray analysis identified an altered expression of myelin-related genes and alcohol-responsive genes in the brain of human alcoholics (Mayfield et al., 2002). Estrogen treatment rapidly upregulates the expression of a battery of estrogen-responsive genes in human breast cancer cells (Wang et al., 2004). These observations suggest that various confounding factors at the time of blood sampling might affect to certain extent the gene expression profile. Since the present study has no detailed information on OTC medications, smoking habits, alcohol intake, and menstrual conditions in MS and CN groups, there exist some limitations in interpreting microarray data. Therefore, further studies on the larger cohort of MS patients and control subjects matched for any potential variables are required to clarify whether the present observations are highly specific for MS, fairly universal in various autoimmune diseases, or closely associated with MS-unrelated confounding factors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbd.2004.10.007.

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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′gatgagaccctttttgctcttcct3′ 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

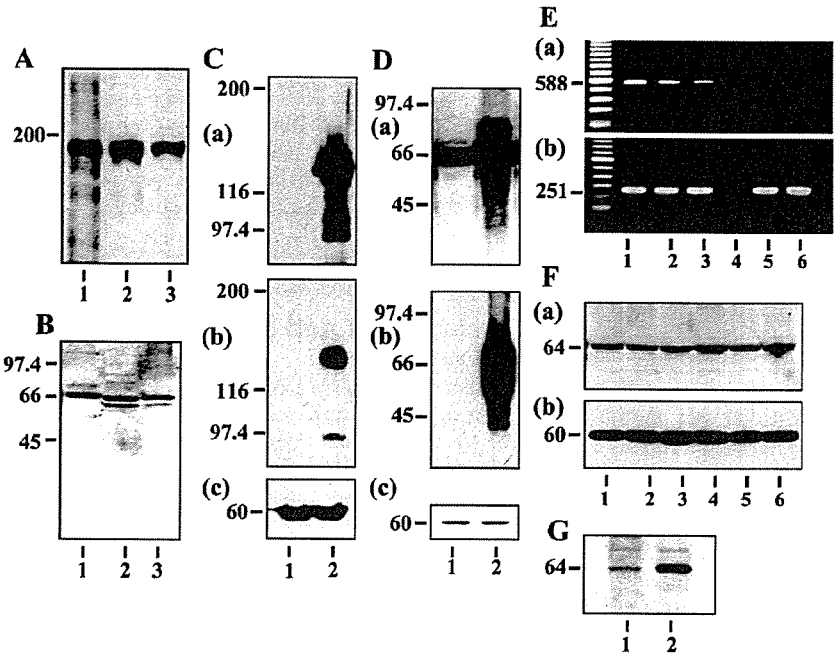

FIGURE 1. Nogo-A and NgR expression in human brain, cultured astrocytes, transfected HEK293 cells. **(A, B)** Immunoblot of brain homogenates with anti-Nogo-A **(A)** or anti-NgR **(B)** antibody. Human (lane 1) or mouse brain (lane 2), and mouse spinal cord (lane 3), 20 μg of protein each. **(C, D)** 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,

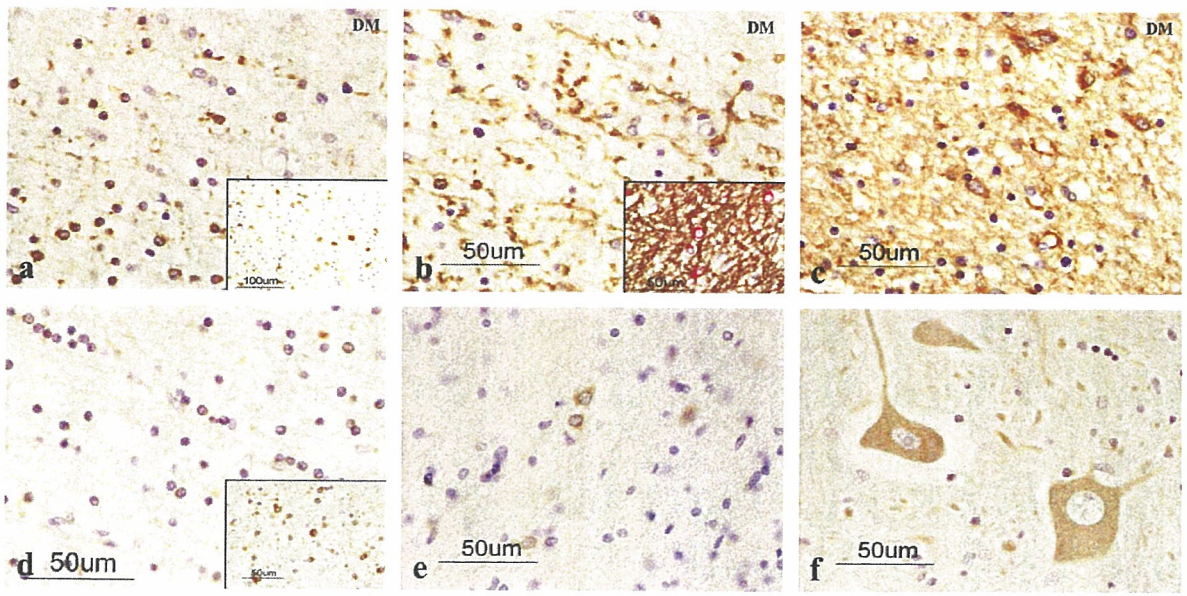

FIGURE 2. Nogo-A expression on oligodendrocytes in demyelinating lesions of MS. Immunohistochemistry. **(a)** No. 744 MS, Nogo-A, the edge of chronic active demyelinating lesions (DM) in the subcortical white matter of the frontal lobe (inset, CD68). **(b)** No. 744 MS, MBP, the adjacent section of panel 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.

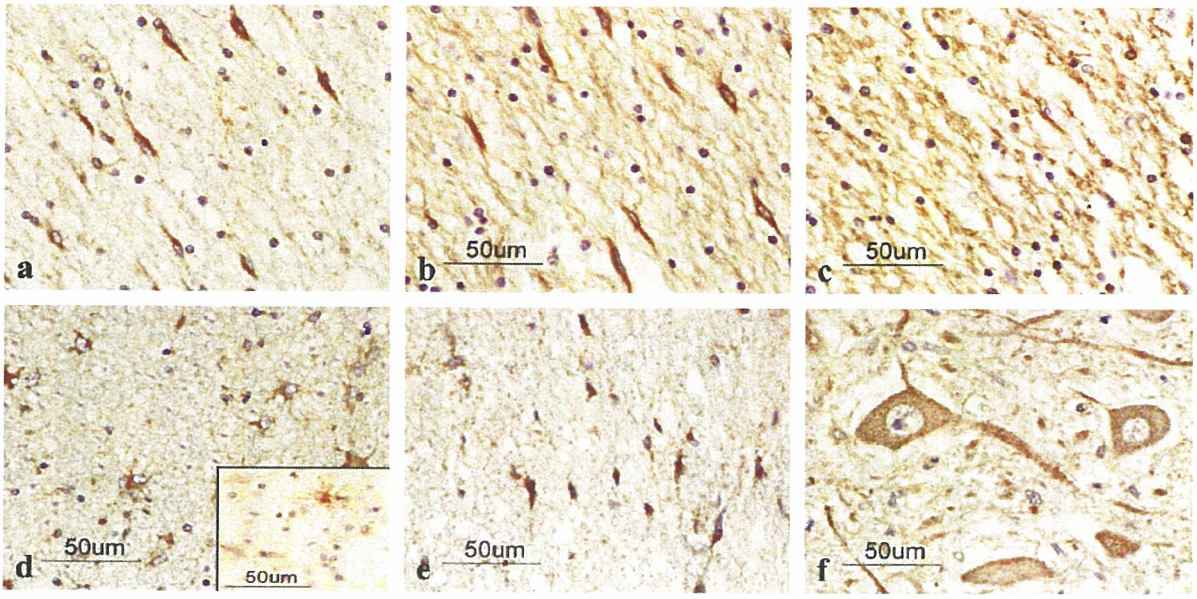

FIGURE 3. NgR expression on reactive astrocytes and microglia in demyelinating lesions of MS. Immunohistochemistry. **(a)** No. 744 MS, NgR, the edge of chronic active demyelinating lesions in the subcortical white matter of the frontal lobe. **(b)** No. 744 MS, GFAP, the adjacent section of panel a. **(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

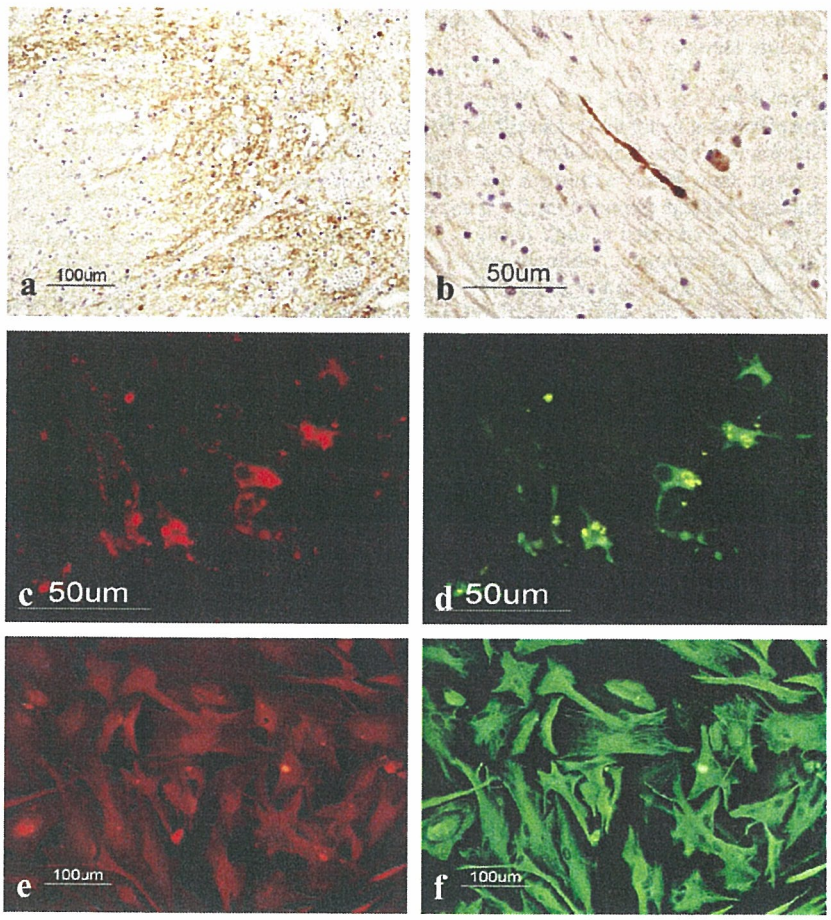

FIGURE 4. Coexpression of NgR and GFAP on reactive astrocytes in MS lesions and cultured human astrocytes. Immunohistochemistry and immunocytochemistry. **(a)** No. 791 MS, $p75^{NTR}$, the substantia gelatinosa in the spinal cord. **(b)** No. 609 MS, APP, chronic active demyelinating lesions in the 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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ORIGINAL ARTICLE

The 14-3-3 Protein Forms a Molecular Complex with Heat Shock Protein Hsp60 and Cellular Prion Protein

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Abstract

The 14-3-3 protein family consists of acidic 30-kDa proteins composed of 7 isoforms expressed abundantly in neurons and glial cells of the central nervous system (CNS). The 14-3-3 protein identified in the cerebrospinal fluid provides a surrogate marker for premortem diagnosis of Creutzfeldt-Jakob disease, although an active involvement of 14-3-3 in the pathogenesis of prion diseases remains unknown. By protein overlay and mass spectrometric analysis of protein extract of NTera2-derived differentiated neurons, we identified heat shock protein Hsp60 as a 14-3-3-interacting protein. The 14-3-3ζ and γ isoforms interacted with Hsp60, suggesting that the interaction is not isoform-specific. Furthermore, the interaction was identified in SK-N-SH neuroblastoma, U-373MG astrocytoma, and HeLa cervical carcinoma cells. The cellular prion protein (PrPC) along with Hsp60 was coimmunoprecipitated with 14-3-3 in the human brain protein extract. By protein overlay, 14-3-3 interacted with both recombinant human Hsp60 and PrPC produced by *Escherichia coli*, indicating that the molecular interaction is phosphorylation-independent. The 14-3-3-binding domain was located in the N-terminal half (NTF) of Hsp60 spanning amino acid residues 27–287 and the NTF of PrPC spanning amino acid residues 23–137. By immunostaining, the 14-3-3 protein Hsp60 and PrPC were colocalized chiefly in the mitochondria of human neuronal progenitor cells in culture, and were coexpressed most prominently in neurons and reactive astrocytes in the human brain. These observations indicate that the 14-3-3 protein forms a molecular complex with Hsp60 and PrPC in the human CNS under physiological conditions and suggest that this complex might become disintegrated in the pathologic process of prion diseases.

Key Words: 14-3-3, Hsp60, Mass spectrometry, Prion protein, Protein overlay.

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INTRODUCTION

The 14-3-3 protein family consists of evolutionarily conserved, acidic 30-kDa proteins composed of seven isoforms named β, γ, ϵ, ζ, η, θ, and σ in eukaryotic cells. A homodimeric or heterodimeric complex composed of the same or distinct isoforms constitutes a large cup-like structure possessing an amphipathic groove with two ligand-binding capacity (1, 2). The dimeric complex acts as a molecular adaptor that interacts with key signaling molecules involved in cell differentiation, proliferation, transformation, and apoptosis. The 14-3-3 protein regulates the function of target proteins by restricting their subcellular location, bridging them to modulate catalytic activity and protecting them from dephosphorylation or proteolysis (3, 4). Although the 14-3-3 protein is widely distributed in neural and nonneural tissues, it is expressed most abundantly in neurons in the central nervous system (CNS), where it represents 1% of total cytosolic proteins (5).

In general, the 14-3-3 protein interacts with phosphoserine-containing motifs of the ligands such as RSXpSXP (mode I) and RXXXpSXP (mode II) in a sequence-specific manner (3, 4). More than 300 proteins, amounting to approximately 0.6% of the human proteome, have been identified as being 14-3-3-binding partners. They include Raf-1 kinase, Bcl-2 antagonist of cell death (BAD), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), cdc25 phosphatase, glycogen synthase kinase-3β (GSK3β), α-synuclein, and ataxin-1 (6–9). Binding of the 14-3-3 protein to Raf-1 is indispensable for its kinase activity in the Ras-MAPK signaling pathway, whereas the interaction of 14-3-3 with BAD, when phosphorylated by a serine/threonine kinase Akt, inhibits apoptosis (1, 2). The target proteins for 14-3-3 are distributed in all subcellular compartments (3, 9). In addition to the phosphorylation-dependent interaction, the 14-3-3 protein occasionally interacts with a set of target proteins in a phosphorylation-independent manner (10–13).

Recently, the 14-3-3 protein detected in the cerebrospinal fluid (CSF) of Creutzfeldt-Jacob disease (CJD) has been used as a biochemical marker that strongly supports the diagnosis of CJD (14, 15). Furthermore, an intense immunoreactivity against the 14-3-3ζ isoform was identified in amyloid plaques in the CJD brain (16). However, it remains unknown whether the 14-3-3 protein plays an active role in the development of prion diseases. The great majority of prion diseases are characterized by intracerebral accumulation of an abnormal prion protein (PrPSc) that is identical in the amino acid sequence to the cellular isoform (PrPC) encoded by the

PRNP gene. PrPSc differs biochemically from PrPC by its β-sheet-enriched structure, detergent insolubility, limited proteolysis by proteinase K, and a slower turnover rate (17). Previous studies suggest that the conversion of PrPC into PrPSc is mediated by a homotypic interaction between endogenous PrPC and incoming or de novo-generated PrPSc through an undefined posttranslational process (17).

Heat shock protein (Hsp) acts as a molecular chaperone that plays an essential role in protein folding, transport, degradation, and signal transduction and is pivotal for recovery from stress-induced protein damage (18). Hsp60, alternatively named as chaperonin 60 (Cpn60), recognizes exposed hydrophobic surfaces of various proteins with globular nonnative conformations, binds them in the central cavity, and stabilizes them against irreversible denaturation and aggregation (19, 20). Dysregulation of Hsp function is supposed to be involved in the pathogenesis of neurodegenerative diseases characterized by an accumulation of intracellular and extracellular protein aggregates (21). A missense mutation in Hsp60 that causes a loss of chaperone function is responsible for an autosomal-dominant form of hereditary spastic paraplegia (22). Several lines of evidence suggest that Hsp60 promotes the conversion of PrPC to PrPSc in vitro, although it remains unknown whether PrPC and Hsp60 could interact in vivo in the same subcellular compartment (23, 24).

The present study, by using protein overlay and mass spectrometry analysis, identified Hsp60 as a 14-3-3-interacting protein in human neurons. Furthermore, PrPC along with Hsp60 was coimmunoprecipitated with 14-3-3 in the human brain protein extract, and they were colocalized chiefly in the mitochondria of human neural cells in culture and coexpressed most prominently in neurons and astrocytes in the human brain. These observations indicate that the 14-3-3 protein forms a molecular complex with Hsp60 and PrPC in the human CNS, and put forth the hypothesis that this complex might become disintegrated during the conversion of PrPC into PrPSc, suggesting a mechanism for elevation of 14-3-3 in the CSF of prion diseases.

MATERIALS AND METHODS

Cell Culture

Human cell lines such as NTera2 teratocarcinoma, SKN-S-H neuroblastoma, U-373MG astrocytoma, HeLa cervical carcinoma, and HEK293 embryonal kidney cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan) and the American Type Culture Collection (Rockville, MD). They were maintained in Dulbecco's Modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (feeding medium). For the induction of neuronal differentiation, NTera2 cells maintained in the undifferentiated state (NTera2-U) were incubated for 4 weeks in feeding medium containing 10^{-5} M *all trans*retinoic acid (Sigma, St. Louis, MO), replated twice, and then plated on a surface coated with Matrigel Basement Membrane Matrix (Becton Dickinson, Bedford, MA). They were incubated for another 2 weeks in feeding medium containing a cocktail of mitotic inhibitors, resulting in the enrichment of differentiated neurons (NTera2-N), as described previously (25).

Human neuronal progenitor (NP) cells isolated from the brain of a human fetus at 18.5 weeks' gestation were obtained from BioWhittaker (Walkersville, MD). NP cells plated on a polyethylenimine-coated surface were incubated in DMEM/F-12 medium containing an insulin–transferrin–selenium (ITS) supplement (Invitrogen), 20 ng/mL recombinant human epidermal growth factor (Higeta, Tokyo, Japan), 20 ng/mL recombinant human basic fibroblast growth factor (PeproTech EC, London, U.K.), and 10 ng/mL recombinant human leukemia inhibitory factor (Chemicon, Temecula, CA) (NP medium), as described previously (26). Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis confirmed the expression of nestin mRNA, a marker for neural stem cells, in NP cells (data not shown).

Two-Dimensional Gel Electrophoresis and Mass Spectrometry Analysis

To prepare total protein extract for 2-dimensional (2D) gel electrophoretic analysis, cells and tissues were homogenized in rehydration buffer composed of 8 M urea, 2% CHAPS, 0.5% carrier ampholytes (pH 4–7 or pH 5–7), 20 mM dithiothreitol, 0.002% bromophenol blue, and a cocktail of protease inhibitors and phosphatase inhibitors (Sigma). Urea-soluble protein was separated by isoelectric focusing (IEF) using the ZOOM IPGRunner system (Invitrogen) loaded with an immobilized pH 4–7 or pH 5.3–6.3 gradient strip, according to the methods described previously (26). After the first dimension of IEF, the protein was separated in the second dimension on a NuPAGE 4% to 12% polyacrylamide gel (Invitrogen). Then, the gel was stained by the Silverquest silver staining kit (Invitrogen) or by the Pro-Q Diamond phosphoprotein gel staining kit that directly labels phosphate groups of the protein attached to serine, threonine, or tyrosine residues in the gel (Molecular Probes, Eugene, OR). For protein overlay analysis, the gel was transferred onto a polyvinylidene difluoride (PVDF) membrane. The spots of interest were excised from the gels, trypsinized, and processed for mass spectrometry (nanoESI-MS/MS) analysis followed by database searching using MASCOT software (Invitrogen Proteome, Yokohama, Japan) (26).

Protein Overlay Analysis

To prepare the probe for protein overlay, the open reading frame of the human 14-3-3ζ isoform gene or 14-3-3γ isoform gene was amplified from cDNA of NTera2-N cells by PCR using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA), and sense and antisense primers listed in the Table. To prepare the full-length, N-terminal, and C-terminal fragments of HSP60 and PrPC, the human HSP60 gene or prion protein gene was amplified by PCR using a panel of primers listed in the Table. The PCR product was cloned into a prokaryotic expression vector pTrcHis-TOPO or pTrcHis2-TOPO (Invitrogen) to produce a fusion protein with an N-terminal Xpress tag or a C-terminal Myc tag. The recombinant proteins were expressed in *Escherichia coli* and purified through a HiTrap

TABLE. Primers Used for Polymerase-Chain Reaction-Based Cloning

Genes	Proteins (amino acid residues)	GenBank Accession No.	Sense Primers
YWHAZ	14-3-3ζ isoform (1–245)	NM_003406	5′atggataaaaatgagctggttcag3′
YWHAZ	14-3-3ζ isoform (1–245)	NM_003406	5′atggataaaaatgagctggttcag3′
YWHAG	14-3-3γ isoform (1–247)	NM_012479	5′atggtggaccgcgagcaactggtg3′
HSPD1	HSP60 full length (1–573)	NM_002156	5′atgcttcggttacccacagtcttt3′
HSPD1	HSP60 N-terminal fragment (27–287)	NM_002156	5′gccaaagatgtaaaatttggtgca3′
HSPD1	HSP60 C-terminal fragment (288–573)	NM_002156	5′ttgaataggctaaaggttggtctt3′
PRNP	cellular prion protein PrPC (23–231)	NM_000311	5′aagaagcgcccgaagcctggaggatggaa3′
PRNP	PrPC N-terminal fragment (23–137)	NM_000311	5′aagaagcgcccgaagcctggaggatggaa3′
PRNP	PrPC C-terminal fragment (138–231)	NM_000311	5′atcatacatttcggcagtgactat3′

Genes	Antisense Primers	Cloning Vector
YWHAZ	5′ttaattttcccctccttctcctgc3′	pTrcHis-TOPO
YWHAZ	5′attttcccctccttctcctgc3′	pTrcHis2-TOPO
YWHAG	5′ttaattgttgccttcgccgccatc3′	pTrcHis-TOPO
HSPD1	5′ttagaacatgccacctcccatac3′	pTrcHis-TOPO
HSPD1	5′ttagacgagtgtacttagagcttc3′	pcDNA4/HisMax-TOPO
HSPD1	5′ttagaacatgccacctcccatac3′	pcDNA4/HisMax-TOPO
PRNP	5′tcagctcgatcctctctggtaataggcctg3′	pTrcHis-TOPO
PRNP	5′tcagggcctgctcatggcacttcc3′	pcDNA4/HisMax-TOPO
PRNP	5′tcagctcgatcctctctggtaataggcctg3′	pcDNA4/HisMax-TOPO

The PCR product was cloned into a prokaryotic expression vector pTrcHis-TOPO or pTrcHis2-TOPO to produce a fusion protein with a Xpress tag or a Myc tag in *E. coli*. It was also cloned into a mammalian expression vector pcDNA4/HisMax-TOPO to express a fusion protein with a Xpress tag in HEK293 cells.

chelating HP column (Amersham Bioscience, Piscataway, NJ) followed by separation on a SDS-PAGE gel. Recombinant human interferon-stimulated protein ISG15 (rhISG15) tagged with Xpress was prepared for a negative control probe, as described previously (26).

The PVDF membrane on which the gel was blotted was incubated at room temperature (RT) overnight with 1 μg/mL recombinant human 14-3-3ζ (rh14-3-3ζ), 14-3-3γ (rh14-3-3γ), or rhISG15, followed by immunolabeling with mouse monoclonal anti-Xpress or anti-Myc antibody (Invitrogen) and HRP-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). After the probes and antibodies were stripped by incubating the membrane at 50°C for 30 minutes in stripping buffer composed of 62.5 mM Tris-HCl (pH 6.7), 2% SDS, and 100 mM 2-mercaptoethanol, the membrane was relabeled with goat anti-HSP60 antibody (N-20; Santa Cruz Biotechnology) followed by incubation with HRP-conjugated anti-goat IgG (Santa Cruz Biotechnology).

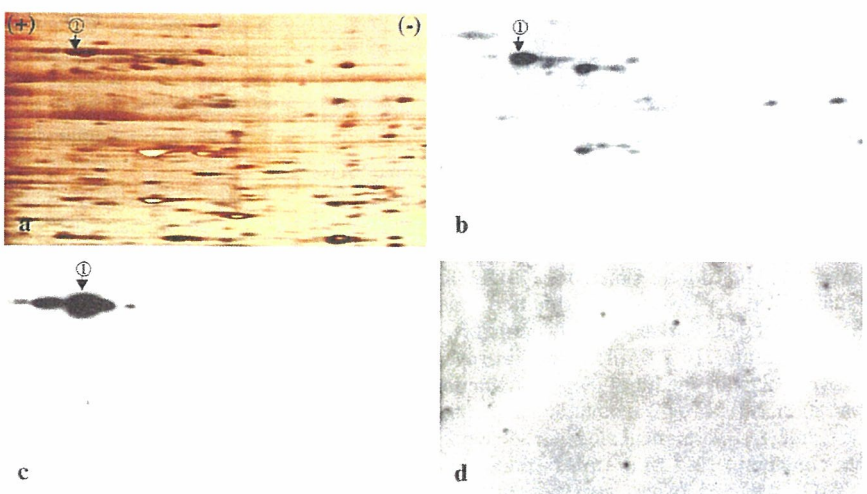

FIGURE 1. Two-dimensional gel electrophoretic analysis of 14-3-3-binding proteins in NTera2-derived differentiated neurons. Two hundred micrograms of total cellular protein isolated from NTera2-derived differentiated neurons (NTera2-N) was separated on a 2-dimensional PAGE gel (pI 5.3-6.3 for isoelectric focusing [IEF] and 4% to 12% for PAGE), silver-stained, transblotted onto a polyvinylidene difluoride (PVDF) membrane, and processed for overlay with recombinant human 14-3-3ζ protein (rh14-3-3ζ) or recombinant human interferon-stimulated protein (rhISG15) tagged with Xpress followed by labeling with anti-Xpress antibody. After the probe and antibody were stripped, the blot was relabeled with anti-Hsp60 antibody. **(a)** Silver staining, **(b)** rh14-3-3ζ, **(c)** Hsp60, **(d)** rhISG15. One major spot labeled with rh14-3-3ζ is named spot 1 (arrow).