研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	ページ	出版年
Satoh J	Protein Microarray Analysis for Rapid Identification of 14-3-3 Protein Binding Partners.	Predki PF	Functional Protein Microarrays in Drug Discovery.	CRC Press	Boca Raton, FL	印刷中	2006

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Satoh J, Nakanishi M, Koike F, Miyake S, Yamamoto T, Kawai M, Kikuchi S, Nomura K, Yokoyama K, Ota K, Kanda T, Fukazawa T, Yamamura T	Microarray analysis identifies an a berrant expression of apoptosis and DNA damage-regulatory genes in multiple sclerosis.	Neurobiology of Disease	18	537-550	2005
Satoh J, Onoue H, Arima K, Yamamura T	Nogo-A and Nogo receptor expression in demyelinating lesions of multiple sclerosis.	Journal of Neuropathology and Experimental Neurology	64 (2)	129-138	2005
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佐藤準一	DNAマイクロアレイによる多発性硬化症の免疫病態の解析. 特集Iサイトカイン・ケモカインからみた多発性硬化症の病型と病態.	Neuroimmunology	13 (2)	167-178	2005
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Oki S, Tomi C, Yamamura T, Miyake S	Preferential Th2 polarization by OCH is supported by incompetent NKT cell induction of CD40L and following production of inflammatory cytokines by bystander cells in vivo.	International Immunology	17 (12)	1619-1629	2005
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Satoh J, Nakanishi M, Koike F, Onoue H, Aranami T, Yamamoto T, Kawai M, Kikuchi S, Nomura K, Yokoyama K, Ota K, Saito T, Ohta M, Miyake S, Kanda T, Fukazawa T, Yamamura T	T cell gene expression profiling identifies distinct subgroups of Japanese multiple sclerosis patients.	Journal of Neuroimmunology		in press	2006

Ⅳ.　研究成果の刊行物・別刷

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Microarray analysis identifies an aberrant expression of apoptosis and DNA damage-regulatory genes in multiple sclerosis

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To clarify the molecular mechanisms underlying multiple sclerosis (MS)-promoting autoimmune process, we have investigated a comprehensive gene expression profile of T cell and non-T cell fractions of peripheral blood mononuclear cells (PBMC) isolated from 72 MS patients and 22 age- and sex-matched healthy control (CN) subjects by using a cDNA microarray. Among 1258 genes examined, 173 genes in T cells and 50 genes in non-T cells were expressed differentially between MS and CN groups. Downregulated genes greatly outnumbered upregulated genes in MS. More than 80% of the top 30 most significant genes were categorized into apoptosis signaling-related genes of both proapoptotic and antiapoptotic classes. They included upregulation in MS of orphan nuclear receptor Nurr1 (NR4A2), receptor-interacting serine/threonine kinase 2 (RIPK2), and silencer of death domains (SODD), and downregulation in MS of TNF-related apoptosis-inducing ligand (TRAIL), B-cell CLL/lymphoma 2 (BCL2), and death-associated protein 6 (DAXX). Furthermore, a set of the genes involved in DNA repair, replication, and chromatin remodeling was downregulated in MS. These results suggest that MS lymphocytes show a complex pattern of gene regulation that represents a counterbalance between promoting and preventing apoptosis and DNA damage of lymphocytes.
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Keywords: Apoptosis; Gene expression profile; Microarray; Multiple sclerosis

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) white matter. Although the etiology of MS remains unknown, immunological studies have suggested that MS is an autoimmune disease mediated by T-lymphocytes secreting proinflammatory T helper type 1 (Th1) cytokines, whose development is triggered by a complex interplay of both genetic and environmental factors (Compston and Coles, 2002). Increasing evidence indicates that the elimination of autoreactive T cells via apoptosis, a common regulatory mechanism for normal development and homeostasis of the immune system, is impaired in MS (Zipp et al., 1999). The mRNA levels of Fas, Fas ligand, and TNF-related apoptosis-inducing ligand (TRAIL) are elevated in peripheral blood mononuclear cells (PBMC) of relapsing–remitting MS (RRMS) patients, while T cell lines established from these patients show a functional defect in the Fas signaling pathway (Comi et al., 2000; Gomes et al., 2003; Huang et al., 2000). The expression of B-cell CLL/lymphoma 2 (BCL2) family proteins is dysregulated in lymphocytes of clinically active MS patients in a manner that promotes resistance to apoptosis (Sharief et al., 2003). Furthermore, apoptosis-regulatory proteins are aberrantly expressed in active MS brain lesions (Bonetti et al., 1999; D'Souza et al., 1996). However, the precise implication of these observations in immunopathogenesis of MS is fairly limited, because most of these studies have focused on a limited range of apoptosis-signaling regulators.

The DNA microarray technology is a novel approach that allows us to systematically and simultaneously monitor the expression of a great number of genes. Application of this

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technique has begun to give us new insights into the complexity of molecular interactions involved in the MS-promoting autoimmune process (Steinman and Zamvil, 2003). Actually, microarray analysis identified upregulation of a set of genes in active MS brain lesions, whose pathological role has not been previously predicted in MS (Lock et al., 2002). Recently, we have studied the gene expression profile of T cells and non-T cells derived from RRMS before and after treatment with interferon-beta (IFNβ) (Koike et al., 2003). IFNβ altered the expression of 21 genes, including nine with IFN-responsive promoter elements, thereby contributing to the therapeutic effects of IFNβ in MS. Supporting our observations, different studies using distinct cDNA microarrays identified IFNβ-responsive genes expressed in PBMC of RRMS patients receiving IFNβ (Stürzebecher et al., 2003; Weinstock-Guttman et al., 2003). Importantly, a recent study showed that a battery of the genes relevant to development of MS include those encoding apoptosis regulators, although this study enrolled only four MS patients (Maas et al., 2002).

Here we investigated a comprehensive gene expression profile of $CD3^+$ T cells and $CD3^-$ non-T cells isolated from 72 MS patients and 22 healthy subjects by using a cDNA microarray containing 1258 genes of various functional classes. We found that 173 genes in T cells and 50 genes in non-T cells were differentially expressed between MS and control (CN) groups. Unexpectedly, more than 80% of the top 30 most significant genes were categorized into apoptosis signaling-related genes of both proapoptotic and antiapoptotic classes, reflecting a counterbalance between resistance and susceptibility of lymphocytes toward apoptosis in MS.

Materials and methods

The study populations

The present study enrolled 72 Japanese, clinically active MS patients and age- and sex-matched 22 Japanese healthy control (CN) subjects. Their demographic characteristics are listed in Table 1. The MS patients were diagnosed according to the established criteria (McDonald et al., 2001). No patients had a past history of treatment with interferons, glatiramer acetate, or mitoxantrone. No patients had received corticosteroids or other immunosuppressants at least 1 month before blood sampling. Written informed consent was obtained from all subjects.

RNA isolation from T cell and non-T cell fractions

Thirty milliliters of heparinized blood was taken in the morning. Within 6 h, PBMCs were isolated by centrifugation on a Ficoll density gradient. Immediately, they were labeled with anti-CD3 antibody-coated magnetic microbeads and separated by AutoMACS (Miltenyi Biotec, Auburn, CA) into a $CD3^+$ T cell fraction and a $CD3^-$ non-T cell fraction, the latter composed of monocytes, B cells, and NK cells. The viability of the cells and the purity of each fraction were verified by trypan blue dye exclusion test and flow cytometric analysis. Total RNA was isolated from each fraction by using RNeasy Mini Kit (Qiagen, Valencia, CA). Five micrograms of purified RNA was in vitro amplified within a linear range of the amplification, and the antisense RNA (aRNA) was processed for cDNA microarray analysis as described previously (Koike et al., 2003).

cDNA microarray analysis

The present study utilized a custom microarray containing duplicate spots of 1258 cDNA immobilized on a poly-L-lysine-coated slide glass (Hitachi Life Science, Kawagoe, Saitama, Japan). They were prepared by PCR of sequence-known genes of various functional classes, including cytokines/growth factors and their receptors, apoptosis regulators, oncogenes, transcription factors, cell cycle regulators, and housekeeping genes. The complete gene list of the microarray is available upon request (express@ls.hitachi.co.jp). Individual aRNA of MS patients and CN subjects was labeled with a fluorescent dye Cy5 by reverse transcriptase reaction. Pooled aRNA of three independent healthy volunteers who were not included in the study was labeled with Cy3 and used as a universal reference to standardize the gene expression levels throughout the experiments as described previously (Koike et al., 2003). The arrays were hybridized at 62°C for 10 h in the hybridization buffer containing equal amounts of Cy3- or Cy5-labeled cDNA, and they were then scanned by the ScanArray 5000 scanner (GSI Lumonics, Boston, MA). The data were analyzed by using the QuantArray software (GSI Lumonics). The average of fluorescence intensities (FI) of duplicate spots was obtained after global normalization between Cy3 and Cy5 signals.

Table 1
Demographic characteristics of the study populations

Characteristics	Multiple sclerosis (MS) patients	Healthy control (CN) subjects
The number of the study population (n)	72	22
Age (average ± SD, year)	36.1 ± 10.3	38.6 ± 12.3
Sex (male vs. female)	17 vs. 55	6 vs. 16
Disease course (RRMS vs. SPMS)	65 vs. 7	(−)
Disease subtype (conventional MS vs. non-conventional MS)	57 vs. 15	(−)
Disease duration (average ± SD, year)	7.7 ± 5.4	(−)
EDSS score (average ± SD, score)	2.8 ± 2.0	(−)
Number of lesions on T2-weighted MRI (average ± SD, number)	24.7 ± 31.9	(−)
Number of relapses during 2 years before blood sampling (average ± SD, number)	1.9 ± 1.5	(−)
Day of IVMP treatment during 2 years before blood sampling (average ± SD, day)	5.9 ± 5.8	(−)
Day of hospitalization during 2 years before blood sampling (average ± SD, day)	49.7 ± 70.0	(−)

Based on the lesion distribution pattern, MS was separated into two subtypes, that is, the conventional MS that affects various regions of the CNS white matter and non-conventional MS that affects chiefly the optic nerve and the spinal cord. Abbreviations: RRMS, relapsing–remitting MS; SPMS, secondary progressive MS; EDSS, expanded disability status scale; and IVMP, intravenous methylprednisolone pulse.

The impact of inter-experiment variability was verified by analyzing a scatter plot. The genes exhibiting the average FI smaller than the level of 1000 were omitted to be processed for further analysis. The gene expression level (GEL) was calculated according to the formula: GEL = FI (Cy5) of the sample/FI (Cy3) of the universal reference. Some results were expressed as box and whisker plots.

The genes were categorized into the group of apoptosis signaling-related genes, when their involvement in regulation of apoptosis was identified through computerized searches in PubMed.

Statistical analysis

The statistical significance of differences in GEL between MS and CN samples was evaluated by a regularized t test (Cyber-T) using the Bayesian inference of variance, where they were considered as significant when the error rate of this test was smaller than 0.05 (Baldi and Long, 2001).

Northern blot analysis

Unfractionated PBMCs of a healthy subject were suspended at 5×10^6 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 55 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were then incubated in a 5%CO_2/95% air incubator at 37°C for 6 h in medium with inclusion of both 25 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO) and 1 μg/ml ionomycin (IOM; Sigma), or incubated for 24 h in the plate coated with 1 μg/ml mouse monoclonal antibody (mAb) against human CD3 (OKT3) or in the medium containing 100 ng/ml recombinant human IFN-gamma (IFNγ) (a specific activity of $\geq 2 \times 10^7$ units/mg, PeproTech, London, UK). They were processed for RNA preparation as described previously (Satoh and Kuroda, 2001). Three micrograms of total RNA was separated on a 1.5% agarose-6% formaldehyde gel and transferred onto a nylon membrane. After prehybridization, the membranes were hybridized at 54°C overnight with the DIG-labeled DNA probe synthesized by the PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany) using the sense and antisense primer sets listed in Supplementary Table 1 online. The specific reaction was visualized on Kodak X-OMAT AR X-ray films by the DIG chemiluminescence detection kit (Roche Diagnostics).

Results

Microarray analysis identified differentially expressed genes in peripheral blood lymphocytes between MS and controls

Among 1258 genes examined, 173 genes in T cell fraction and 50 genes in non-T cell fraction were expressed differentially between 72 MS patients and 22 CN subjects (see Supplementary Table 2 online for all data set). In T cell fraction, 25 genes were upregulated, while 148 genes were downregulated in MS. In non-T cell fraction, 11 genes were upregulated, while 39 genes were downregulated in MS. Thus, downregulated genes greatly outnumbered upregulated genes in MS. No genes showed an opposed pattern of regulation between T cell and non-T cell fractions. The top 30 most significant genes are listed in Tables 2 and 3, and among them, top 10 are expressed as box and whisker plots (Figs. 1 and 2). Among top 30 genes, six genes, such as regulator of G protein signaling 14 (RGS14), SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 3 (SMARCA3), transcription factor 17 (TCF17), carbohydrate sulfotransferase 4 (CHST4), cytochrome c oxidase assembly protein (COX15), and death-associated protein 6 (DAXX), were downregulated coordinately in both cell fractions.

The majority of top 30 differentially expressed genes between MS and controls were categorized into apoptosis signaling-related genes

In T cell fraction, the top 30 contained 25 genes closely related to apoptosis signaling (Table 2). They included upregulation in MS of nuclear receptor subfamily 4, group A, member 2 (NR4A2; No. 1), transcription factor 8 (TCF8; No. 2), and cytochrome $P450$ family 1, subfamily A, polypeptide 2 (CYP1A2; No. 3). They also included downregulation in MS of RGS14 (No. 4), mitogen-activated protein kinase 1 (MAPK1; No. 6), SMARCA3 (No. 7), TCF17 (No. 9), heat shock 70-kD protein 1A (HSPA1A; No. 10), TRAIL (No. 12), topoisomerase I (TOP1; No. 13), protein tyrosine phosphatase, non-receptor type 6 (PTPN6; No. 14), chemokine, CC motif, receptor 5 (CCR5; No. 15), v-erb-a erythroblastic leukemia viral oncogene homolog 4 (ERBB4; No. 17), transcription factor 21 (TCF21; No. 18), ATPase, hydrogen-transporting, lysosomal, 56/58 kDa, V1 subunit B, isoform 2 (ATP6V1B2; No. 19), cAMP responsive element-binding protein 1 (CREB1; No. 20), integrin, beta 1 (ITGB1; No. 21), COX15 (No. 22), Myc protooncogene (MYC; No. 23), BCL2-associated athanogene 1 (BAG1; No. 24), cell division cycle 16 (CDC16, No. 25), DAXX (No. 27), TGFβ-stimulated gene 22 (TSC22; No. 28), GA-binding protein transcription factor, beta subunit 1 (GABPB1; No. 29), and poly(ADP-ribose) polymerase (PARP; No. 30). Surprisingly, the top 30 included none of Th1-specific marker genes except for CCR5. The concurrent downregulation of proapoptotic and antiapoptotic genes such as TRAIL, DAXX, and BAG1 suggests that the gene expression pattern in T cells of MS represents a counterbalance between promoting and preventing apoptosis.

In non-T cell fraction, the top 30 contained 27 apoptosis signaling-related genes (Table 3). They included upregulation in MS of cell division cycle 42 (CDC42; No. 2), receptor-interacting serine/threonine kinase 2 (RIPK2; No. 3), Max dimerization protein (MAD; No. 5), chemokine, CXC motif, ligand 2 (CXCL2; No. 6), silencer of death domains (SODD; No. 7), topoisomerase 2 alpha (TOP2A; No. 8), and intercellular adhesion molecule-1 (ICAM1; No. 1). ICAM1 was listed as an apoptosis signaling-related gene because it provides a costimulatory signal to protect T cells from apoptosis by upregulation of BCL2 (Kohlmeier et al., 2003). They also included downregulation in MS of SMARCA3 (No. 9), RGS14 (No. 10), COX15 (No. 11), A-kinase anchor protein 11 (AKAP11; No. 12), TCF17 (No. 13), cell division cycle 25B (CDC25B; No. 14), granzyme A (GZMA; No. 15), BCL2 (No. 17), complement component receptor 2 (CR2; No. 18), replication protein A1 (RPA1; No. 19), RNA polymerase II, subunit H (POLR2H; No. 20), E2F transcription factor 5 (E2F5; No. 21), Ras associated protein RAB7-like 1 (RAB7L1; No. 22), nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3 (NFATC3; No. 23), heat shock 70-kD protein-like 1 (HSPA1L; No. 24), retinoblastoma-binding protein 4 (RBBP4; No.

Table 2
Top 30 genes expressed differentially in T cells between MS and controls

No.	Symbol	GenBank accession number	Description	Presumed function	Possible involvement in apoptosis regulation	Significance (p-log)
The upregulated genes						
1	NR4A2	NM_006186	Nuclear receptor subfamily 4, group A, member 2	an orphan nuclear receptor of the steroid–thyroid hormone receptor superfamily designated Nurr1	(+)	2.55E−12
2	TCF8	NM_030751	Transcription factor 8	a transcription repressor for IL-2 expression in T cells designated ZEB	(+)	1.17E−09
3	CYP1A2	NM_000761	Cytochrome P450, family 1, subfamily A, polypeptide 2	a monooxygenase involved in the metabolism of drugs, toxic chemicals, and carcinogens	(+)	1.64E−08
The downregulated genes						
4	RGS14	NM_006480	Regulator of G protein signaling 14	a downregulator of signaling through G protein-coupled receptors	(+)	1.51E−13
5	CHST2	NM_004267	Carbohydrate sulfotransferase 2	an *N*-acetylglucosamine-6-*O*-sulfotransferase	unknown	6.43E−13
6	MAPK1	NM_002745	Mitogen-activated protein kinase 1	a protein kinase designated ERK2 (p42) that regulates diverse cellular functions	(+)	6.02E−12
7	SMARCA3	NM_003071	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 3	a DNA helicase-like chromatin remodeling enzyme	(+)	1.70E−11
8	TPST2	NM_003595	Tyrosylprotein sulfotransferase 2	a tyrosylprotein sulfotransferase	unknown	2.31E−11
9	TCF17	NM_005649	Transcription factor 17 designated Kid-1	a transcriptional repressor of renal genes	(+)	3.14E−11
10	HSPA1A	NM_005345	Heat shock 70kD protein 1A	an inducible member of the HSP70 family	(+)	4.67E−11
11	AGTRL2	NM_005162	Angiotensin receptor-like 2	a protein homologous to the angiotensin II receptor type 1	unknown	3.51E−10
12	TRAIL	NM_003810	TNF-related apoptosis-inducing ligand	an apoptosis-inducing ligand of the TNF family for DR4 and DR5	(+)	5.19E−10
13	TOP1	NM_003286	Topoisomerase 1	a DNA topoisomerase	(+)	7.03E−10
14	PTPN6	NM_080549	Protein tyrosine phosphatase, non-receptor type 6	a protein tyrosine phosphatase with SH2 domains designated SHP-1	(+)	7.77E−10
15	CCR5	NM_000579	Chemokine, CC motif, receptor 5	a chemokine receptor for RANTES, MIP1α, MIP1β and MCP2	(+)	1.10E−09
16	CHST4	NM_005769	Carbohydrate sulfotransferase 4	an *N*-acetylglucosamine 6-O sulfotransferase	unknown	1.84E−09
17	ERBB4	NM_005235	V-erb-a erythroblastic leukemia viral oncogene homolog 4	an EGF receptor-related receptor tyrosine kinase interacting with neuregulins	(+)	2.22E−09
18	TCF21	NM_003206	Transcription factor 21	a transcription factor designated Capsulin	(+)	4.99E−09
19	ATP6V1B2	NM_001693	ATPase, hydrogen-transporting, lysosomal, 56/58kD, V1 subunit B, isoform 2	a vacuolar ATPase that mediates acidification of intracellular organelles	(+)	5.10E−09
20	CREB1	NM_134442	Cyclic AMP responsive element-binding protein 1	a CRE-binding transcription factor for cAMP-responsive genes	(+)	6.58E−09
21	ITGB1	NM_002211	Integrin, beta-1	a common beta chain of the VLA protein family	(+)	7.16E−09
22	COX15	NM_078470	Cytochrome c oxidase assembly protein COX15	a protein essential for assembly of COX	(+)	1.13E−08
23	MYC	NM_002467	Myc protooncogene	a transcription factor that regulates diverse cellular functions	(+)	1.18E−08
24	BAG1	NM_004323	BCL2-associated athanogene 1	an enhancer of the antiapoptotic effects of BCL2	(+)	1.51E−08
25	CDC16	NM_003903	Cell division cycle 16	a component of the anaphase-promoting complex essential for mitosis	(+)	1.99E−08
26	SLC35A1	NM_006416	Solute carrier family 35, member A1	a CMP-sialic acid transporter	unknown	2.06E−08
27	DAXX	NM_001350	Death-associated protein 6	a protein that interacts with the death domain of Fas	(+)	2.23E−08
28	TSC22	NM_006022	TGFβ-stimulated gene 22	a transcription factor induced by TGFβ	(+)	2.34E−08
29	GABPB1	NM_005254	GA-binding protein transcription factor, beta subunit 1	a nuclear transcription factor for cytochrome *c* oxidase	(+)	6.16E−08
30	PARP	NM_001618	Poly(ADP-ribose) polymerase	a chromatin-associated enzyme that catalyzes polyADP-ribosylation of nuclear proteins	(+)	6.72E−08

The genes were categorized into the group of apoptosis signaling-related genes, when their involvement in regulation of apoptosis was identified through computerized searches in PubMed. The average signal intensity and average increase (fold change) of the genes listed are shown in Supplementary Table 2 online.

Table 3
Top 30 genes expressed differentially in non-T cells between MS and controls

No.	Symbol	GenBank accession number	Description	Presumed function	Possible involvement in apoptosis regulation	Significance (p-log)
The upregulated genes						
1	ICAM1	NM_000201	Intercellular adhesion molecule-1	a cell surface glycoprotein ligand (CD54) for LFA-1 and Mac-1	(+)	1.11E−09
2	CDC42	NM_001791	Cell division cycle 42	a small GTPase that regulates diverse cellular functions	(+)	1.49E−08
3	RIPK2	NM_003821	Receptor-interacting serine/threonine kinase 2	a protein kinase interacting with CLARP in the Fas-signaling pathway	(+)	1.88E−07
4	IL1R2	NM_004633	IL-1 receptor, type II	a decoy receptor for IL-1 that inhibits IL-1 activity	unknown	4.56E−07
5	MAD	NM_002357	Max dimerization protein	a transcriptional repressor that competes with MYC for binding to MAX	(+)	1.00E−06
6	CXCL2	NM_002089	Chemokine, CXC motif, ligand 2	a chemokine designated MIP2 binding to CXCR2	(+)	1.91E−06
7	SODD	NM_004874	Silencer of death domains	an adaptor protein designated BAG4 associated with HSP70 and the death domain of TNFR1 and DR3	(+)	3.13E−06
8	TOP2A	NM_001067	Topoisomerase 2 alpha	a DNA topoisomerase	(+)	4.82E−06
The downregulated genes						
9	SMARCA3	NM_003071	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 3	a DNA helicase-like chromatin remodeling enzyme	(+)	3.95E−08
10	RGS14	NM_006480	Regulator of G protein signaling 14	a downregulator of signaling through G protein-coupled receptors	(+)	5.44E−08
11	COX15	NM_078470	Cytochrome c oxidase assembly protein COX15	a protein essential for assembly of COX	(+)	6.43E−08
12	AKAP11	NM_016248	A-kinase anchor protein 11	a protein anchoring the regulatory subunit of protein kinase A	(+)	1.68E−07
13	TCF17	NM_005649	Transcription factor 17	a transcriptional repressor of renal genes designated Kid-1	(+)	1.92E−07
14	CDC25B	NM_021874	Cell division cycle 25B	a tyrosine phosphatase that activates the cyclin dependent kinase CDC2	(+)	2.40E−07
15	GZMA	NM_006144	Granzyme A	a cytotoxic T cell- and NK cell-specific serine protease	(+)	2.49E−07
16	CHST4	NM_005769	Carbohydrate sulfotransferase 4	an *N*-acetylglucosamine 6-O sulfotransferase	unknown	3.46E−06
17	BCL2	NM_000633	B-cell CLL/lymphoma 2	a mitochondrial membrane protein that blocks the apoptotic death	(+)	4.81E−07
18	CR2	NM_001877	Complement component receptor 2	a membrane receptor (CD21) for C3d	(+)	5.88E−07
19	RPA1	NM_002945	Replication protein A1	a single-stranded DNA-binding protein that regulates DNA replication	(+)	6.72E−07
20	POLR2H	NM_006232	RNA polymerase II, subunit H	a subunit of RNA polymerase II	(+)	7.28E−07
21	E2F5	NM_001951	E2F transcription factor 5	a transcription factor of the E2F family	(+)	1.00E−06
22	RAB7L1	NM_003929	Ras associated protein RAB7-like 1	a RAS-related small GTP-binding protein	(+)	1.49E−06
23	NFATC3	NM_173165	Nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3	a component of DNA-binding transcription complex that regulates the gene expression in T cells	(+)	1.66E−06
24	HSPA1L	NM_005527	Heat shock 70-kD protein-like 1	a constitutive member of the HSP70 family	(+)	1.87E−06
25	RBBP4	NM_005610	Retinoblastoma-binding protein 4	a nuclear protein binding to RB1	(+)	3.13E−06
26	PRKDC	NM_006904	Protein kinase, DNA-activated, catalytic subunit	a nuclear serine/threonine protein kinase	(+)	3.36E−06
27	RASSF1	NM_170714	Ras association domain family 1	a lung tumor suppressor gene having a Ras-association domain	(+)	3.49E−06
28	DAXX	NM_001350	Death-associated protein 6	a protein that interacts with the death domain of Fas	(+)	5.16E−06
29	EGF	NM_001963	Epidermal growth factor	a potent mitogenic factor for the cells of both ectodermal and mesodermal origin	(+)	5.74E−06
30	NPR2L	NM_006545	Nitrogen permease regulator 2-like	a possible tumor suppressor gene	unknown	1.13E−05

The genes were categorized into the group of apoptosis signaling-related genes, when their involvement in regulation of apoptosis was identified through computerized searches in PubMed. The average signal intensity and average increase (fold change) of the genes listed are shown in Supplementary Table 2 online.

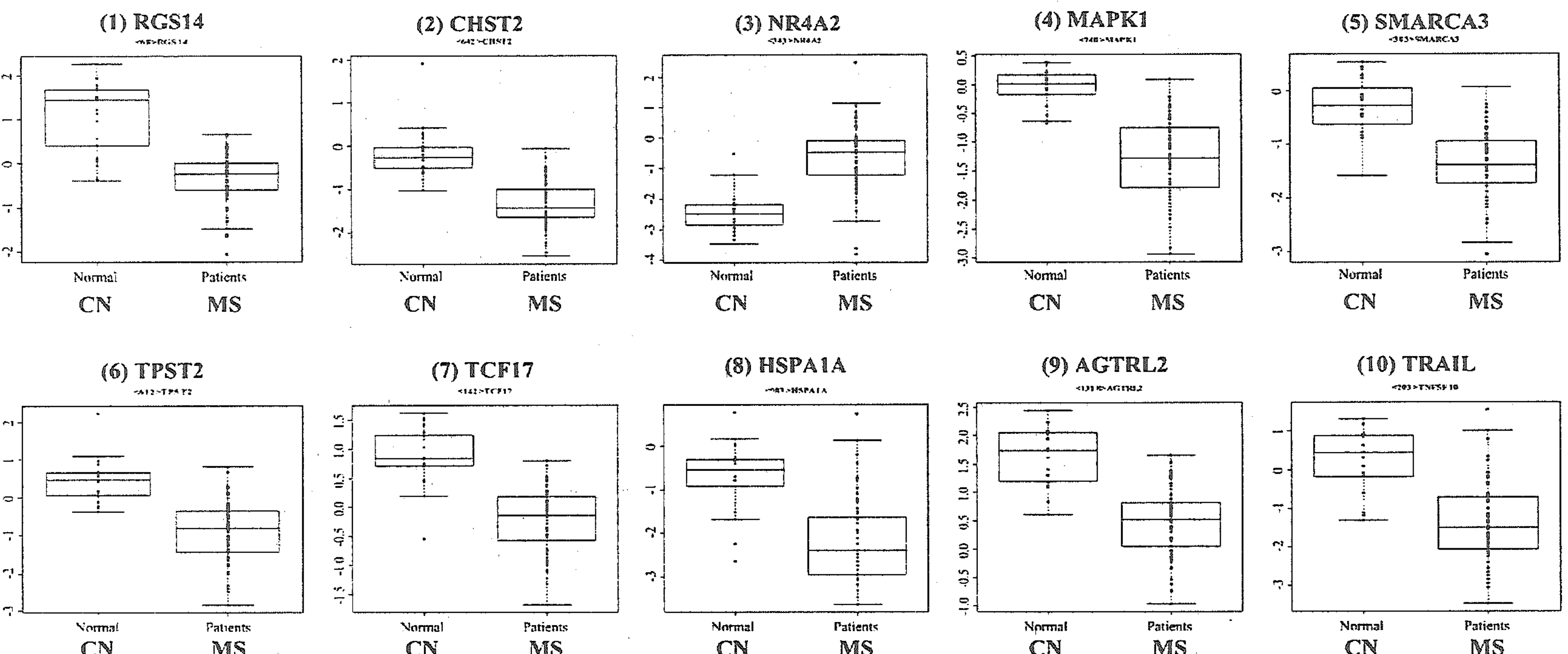

Fig. 1. Top 10 differentially expressed genes in T cell fraction between MS and CN groups. The gene expression profile was studied in $CD3^+$ T cell fraction isolated from 72 MS patients and 22 healthy control (CN) subjects by analyzing a cDNA microarray containing 1258 genes. RNA of MS and CN samples was labeled with Cy5, while RNA of a universal reference was labeled with Cy3. The genes differentially expressed between both groups were identified by Cyber-T test. Top 10 significant genes are shown as box and whisker plots where the longitudinal axis indicates log gene expression level (GEL). They are arranged in order of the significance listed in Table 2. The plots represent the following genes: (1) regulator of G protein signaling 14 (RGS14), (2) carbohydrate sulfotransferase 2 (CHST2), (3) nuclear receptor subfamily 4, group A, member 2 (NR4A2), (4) mitogen-activated protein kinase 1 (MAPK1), (5) SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 3 (SMARCA3), (6) tyrosylprotein sulfotransferase 2 (TPST2), (7) transcription factor 17 (TCF17), (8) heat shock 70-kDa protein 1A (HSPA1A), (9) angiotensin receptor-like 2 (AGTRL2), and (10) TNF-related apoptosis-inducing ligand (TRAIL).

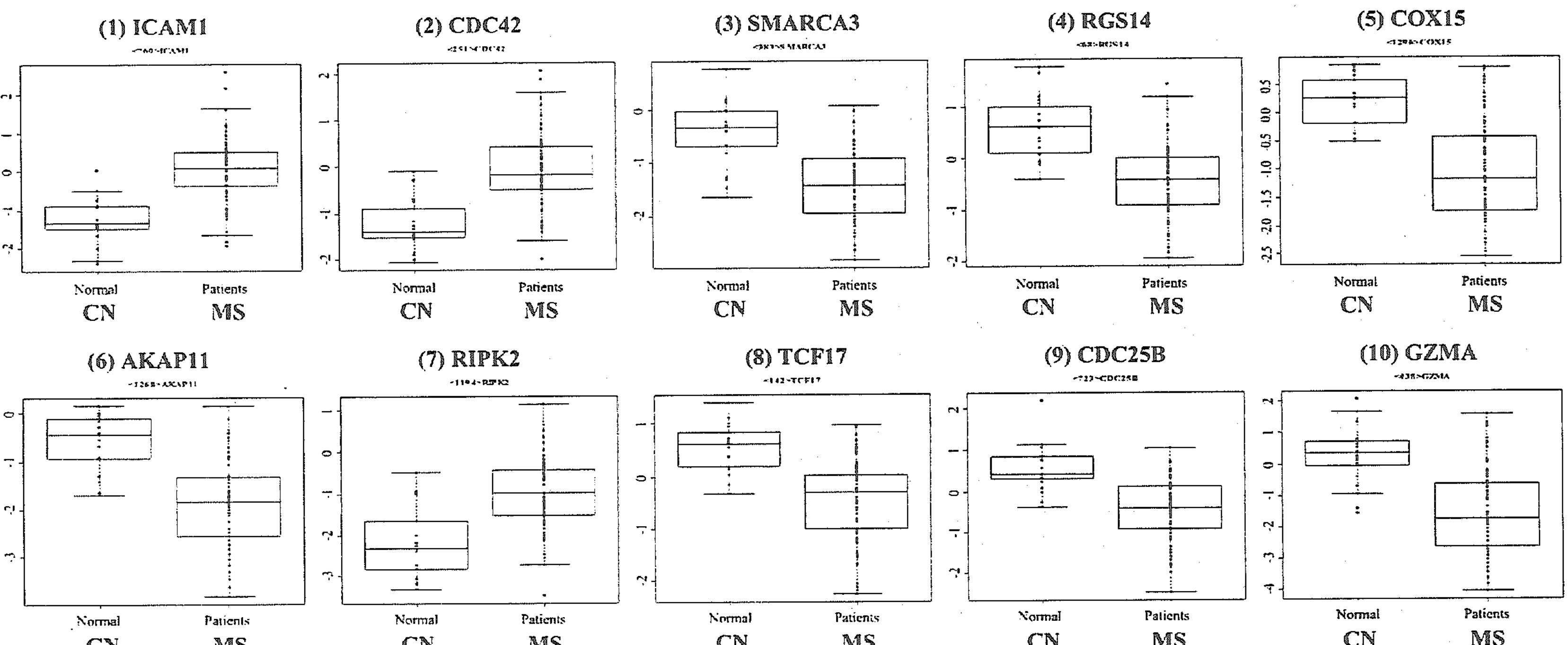

Fig. 2. Top 10 differentially expressed genes in non-T cell fraction between MS and CN groups. The gene expression profile was studied in $CD3^-$ non-T cell fraction isolated from 72 MS patients and 22 CN subjects by analyzing 1258 cDNA microarrays. See Fig. 1. Top 10 genes are shown as box and whisker plots where the longitudinal axis indicates log GE. They are arranged in order of the significance listed in Table 3. The plots represent the following genes: (1) intercellular adhesion molecule-1 (ICAM1), (2) cell division cycle 42 (CDC42), (3) SMARCA3, (4) RGS14, (5) cytochrome *c* oxidase assembly protein (COX15), (6) A-kinase anchor protein 11 (AKAP11), (7) receptor-interacting serine/threonine kinase 2 (RIPK2), (8) TCF17, (9) cell division cycle 25B (CDC25B), and (10) granzyme A (GZMA).

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

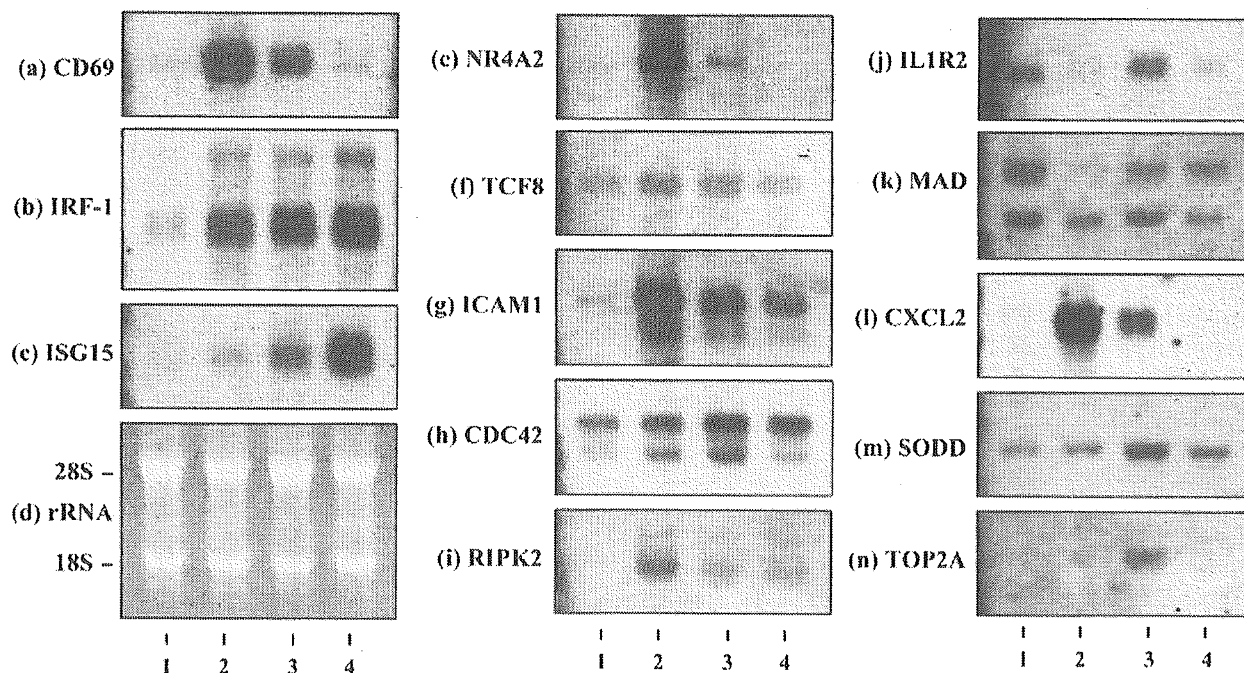

Fig. 3. The genes upregulated in MS were expressed in cultured PBMC in an activation-dependent manner. Unfractionated PBMCs of a healthy subject were incubated for 6 h in medium without (lane 1) or with inclusion of 25 ng/ml PMA and 1 μg/ml IOM (lane 2), or for 24 h in the plate coated with 1 μg/ml anti-CD3 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.

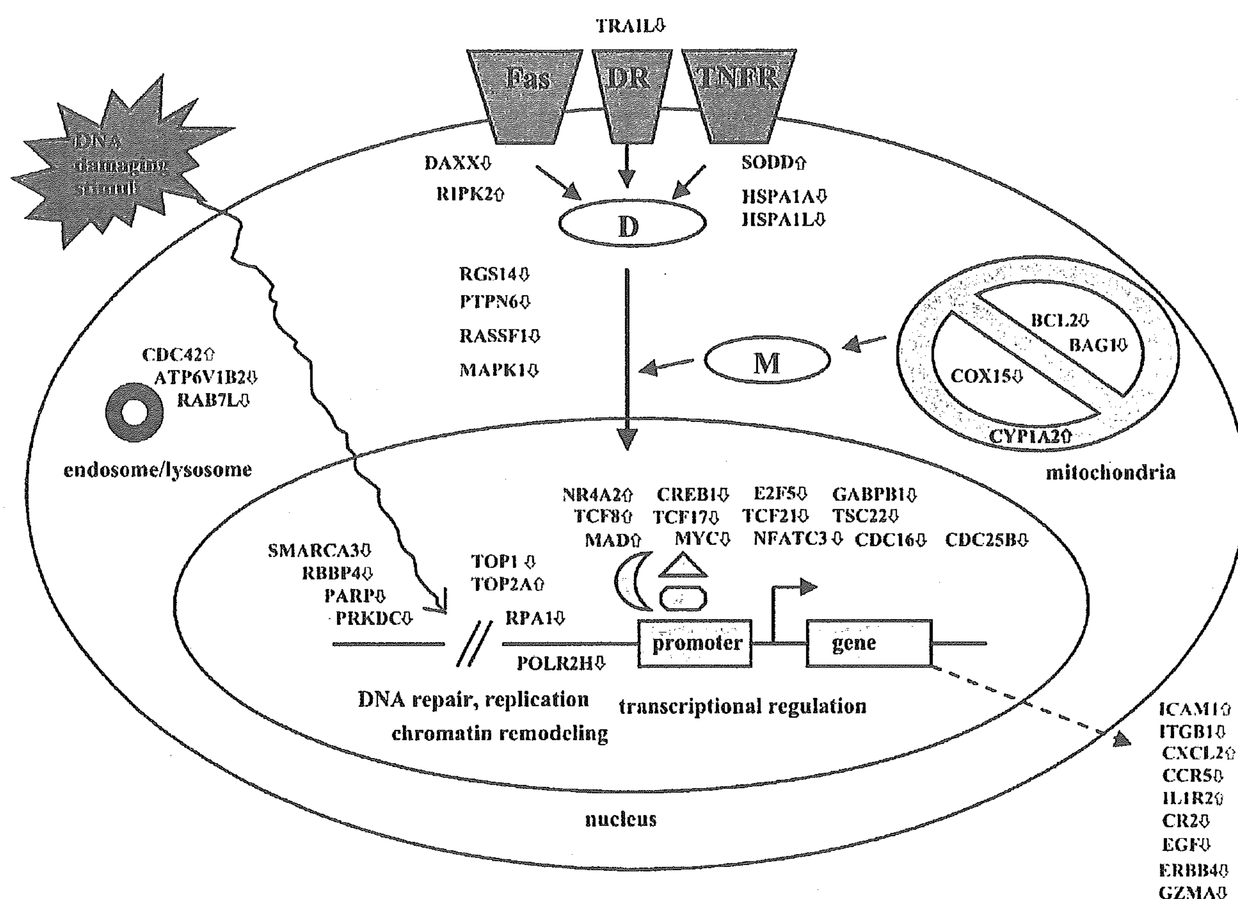

Fig. 4. Aberrant expression of apoptosis signaling-related genes in MS lymphocytes. More than 80% of the top 30 differentially expressed genes between MS and CN groups were categorized into apoptosis signaling-related genes of both proapoptotic and antiapoptotic classes, whose expression was either upregulated (⇑, 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 *P450* 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.

Acknowledgments

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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.

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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.

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