リンを合成するのみならず，代謝もしうることが発見されたことから，DAOの少ない前脳において，セリンラセマーゼがD-セリン濃度調節に関与している可能性が提唱されている.[18] しかし，統合失調症のゲノム解析からは当疾患とセリンラセマーゼ遺伝子との関連についての報告はなされていない.

統合失調症の発症と病態における責任病巣は大脳であることが予想されるため，統合失調症患者の有症期において，D-セリン濃度が減少している可能性，もしくはDAOの大脳における発現や活性が相対的に亢進している可能性が考えられる．また，細胞によるD-アミノ酸取り込み機構，DAOの発現調節因子や細胞内シグナリングにおける位置付け等D-アミノ酸・DAOの生体内における役割については，まだこれから解明すべき点が山積している.

7 おわりに

当研究室は，DAOタンパクの結晶化と3次元構造解析をはじめとするDAO構造・活性相関の解明と酵素の活性アッセイシステムの確立を通じて，NMDA受容体機能異常に基づく難治性精神疾患の治療へ貢献することを目標として研究を行っている．さらに脳の発生・分化の過程や中枢神経疾患の病態におけるD-セリンとDAOの生理的・病理的意義の解明も目指している．近年その存在が明らかになり，情報が蓄積されるにつれ急速にその重要性を増してきた「D-アミノ酸制御システム」の解明は，難治性精神疾患をはじめとする疾患治療に対する新たなアプローチを可能にし，医学的応用面において大いに貢献することが期待される.

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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.

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 *P*450 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 1 (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.

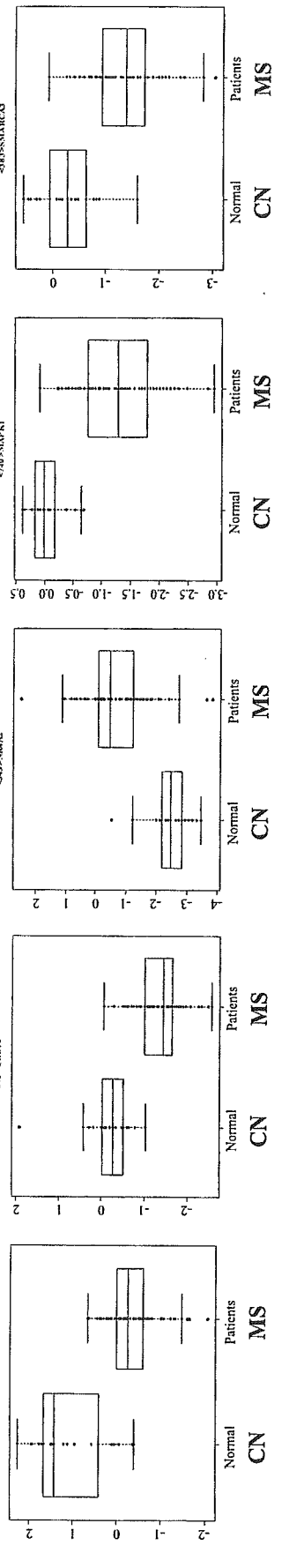

Fig. 1. Top 10 differentially expressed genes in T cell fraction between MS and CN groups. The gene expression profile was studied in $CD3^+$ T cell fraction isolated from 72 MS patients and 22 healthy control (CN) subjects by analyzing a cDNA microarray containing 1258 genes. RNA of MS and CN samples 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).

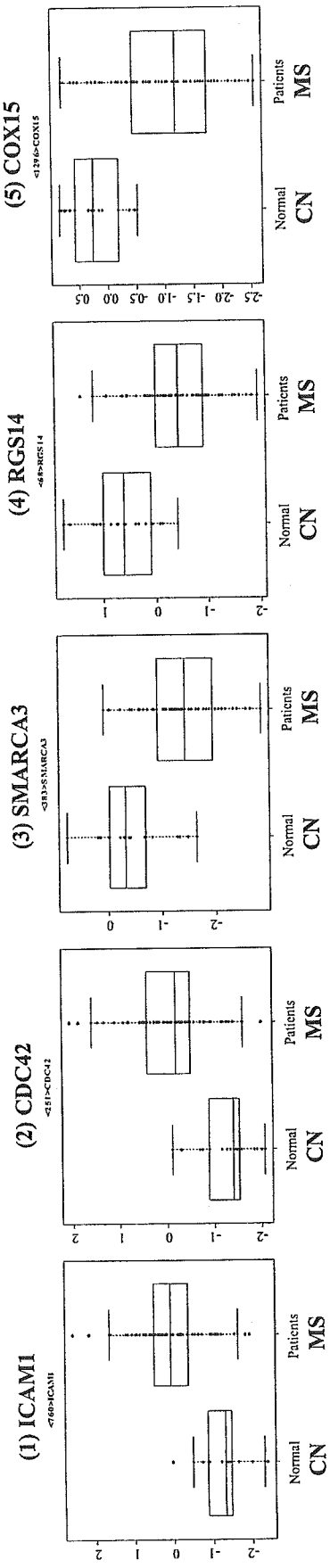

Fig. 2. Top 10 differentially expressed genes in non-T cell fraction between MS and CN groups. The gene expression profile was studied in $CD3^-$ non-T cell fraction isolated from 72 MS patients and 22 CN subjects by analyzing 1258 cDNA microarrays. See Fig. 1. Top 10 genes are shown as box and whisker 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

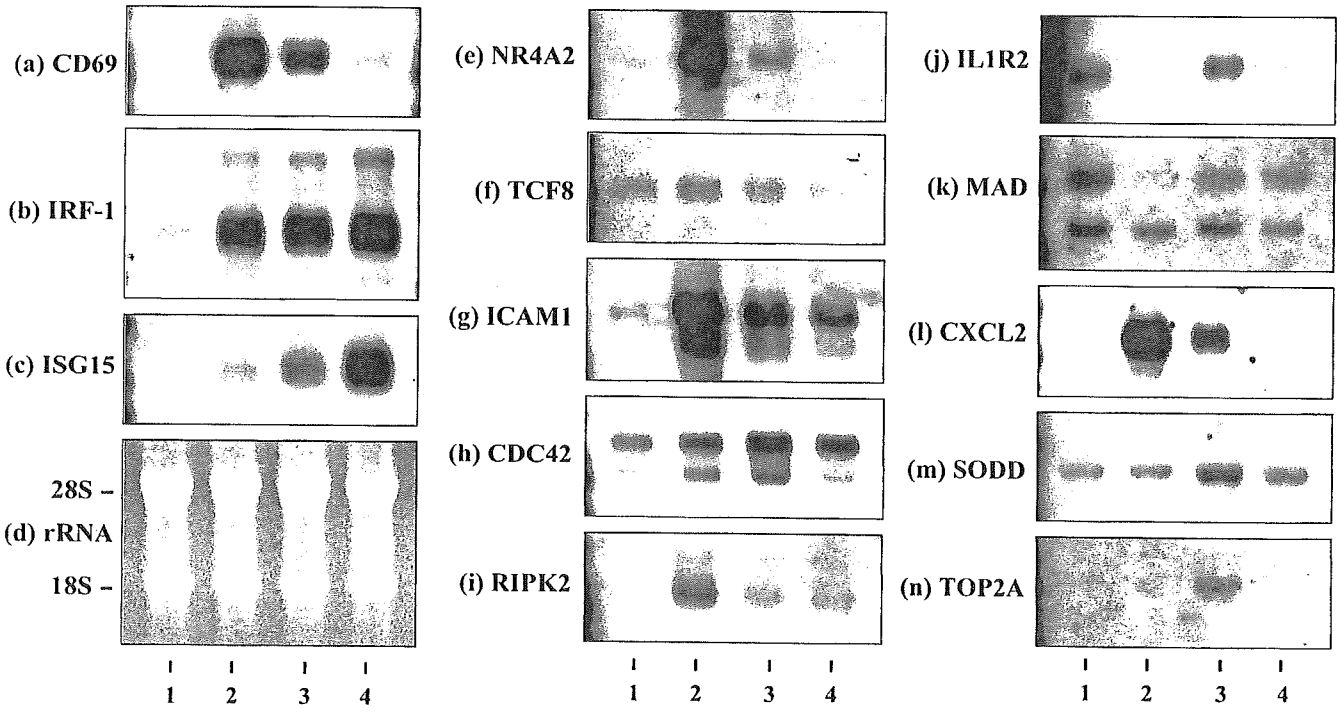

Fig. 3. The genes upregulated in MS were expressed in cultured PBMC in an activation-dependent manner. Unfractionated PBMCs of a healthy subject were incubated for 6 h in medium without (lane 1) or with inclusion of 25 ng/ml PMA and 1 μg/ml IOM (lane 2), or for 24 h in the plate coated with 1 μg/ml anti-CD3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

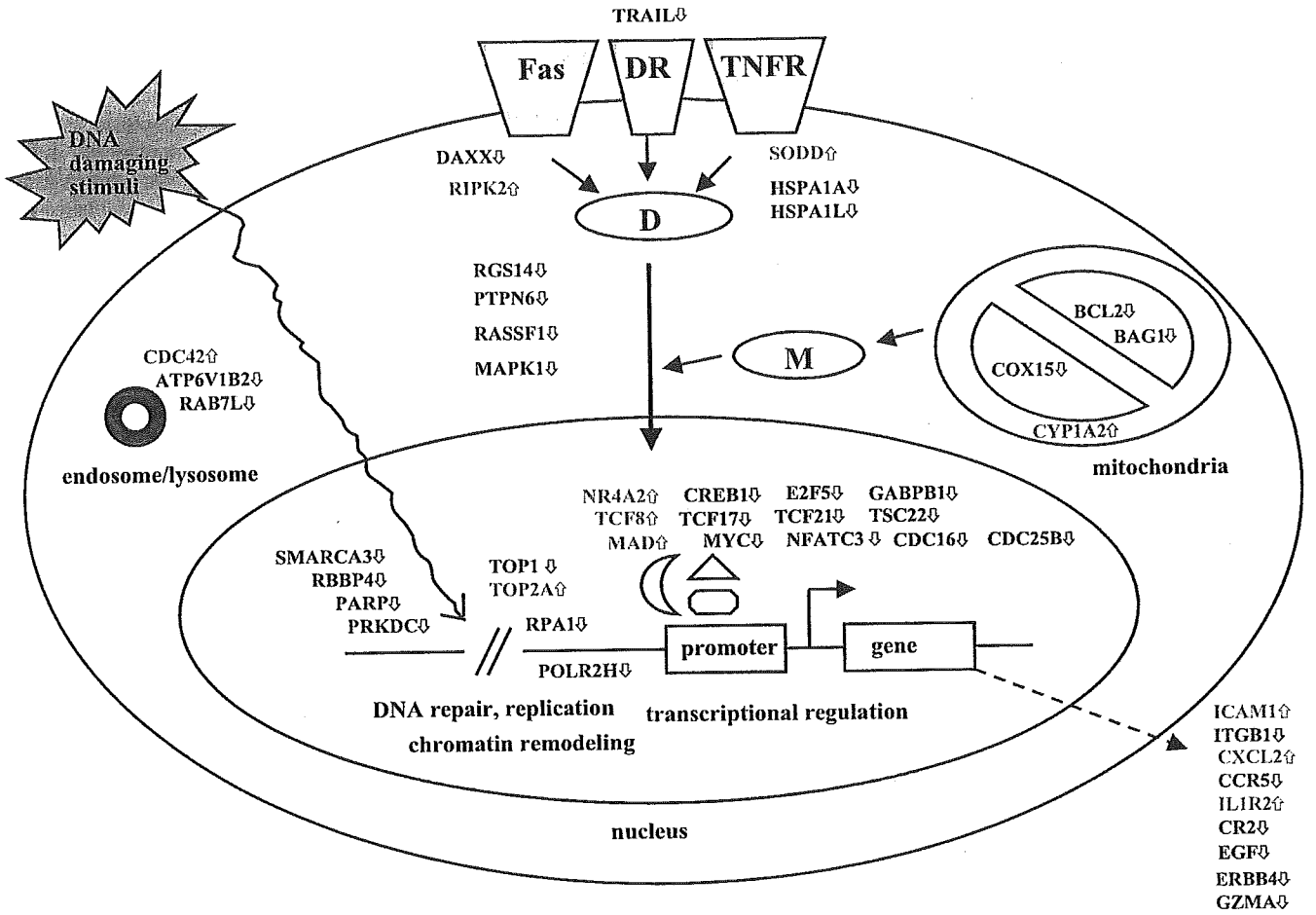

Fig. 4. Aberrant expression of apoptosis signaling-related genes in MS lymphocytes. More than 80% of the top 30 differentially expressed genes between MS and CN groups were categorized into apoptosis signaling-related genes of both proapoptotic and antiapoptotic classes, whose expression was either upregulated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

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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原　著

Pioglitazone長期投与による筋強直性ジストロフィーの糖尿病治療

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要旨：筋強直性ジストロフィー（DM1）に合併する糖尿病に対しインスリン抵抗性改善薬pioglitazoneの効果を検討した．糖尿病を合併したDM1患者8人で，うち3人はglibenclamideを内服していたが血糖コントロールは不良であった．Pioglitazone 15mg連日投与（glibenclamideは中止）で平均15.4±9.6カ月間観察し，血糖コントロールは良好で，低血糖発作や副作用は生じなかった．75g経口ブドウ糖負荷試験では，糖負荷後の血糖は全例で低下し，インスリン分泌量は4人がほぼ不変，4人が低下した．DM1患者の糖尿病には，とくに反応性にインスリンが過分泌の傾向にある患者では，pioglitazoneが有効であると考えた．

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Key words：筋強直性ジストロフィー，糖尿病，インスリン抵抗性，チアゾリジン誘導体，75g糖負荷試験

はじめに

筋強直性ジストロフィー（DM1）では5～12.5%の患者が糖尿病を合併する[1]．糖尿病にいたらなくとも，しばしばインスリン抵抗性を合併し，空腹時の血糖や血中インスリン値（IRI）が正常であっても，ブドウ糖負荷によりいちじるしくIRIが上昇することが多い[2)~5)]．DM1の糖尿病は2型糖尿病と類似した病態であるが[6)7)]，2型糖尿病でおこなわれる食事療法と運動療法は十分におこなえないことが多い．DM1患者は，筋力低下のため自分で食事が作れず，腹部症状や欠食も多く，食事療法は乱れやすい．運動療法は筋力低下のために困難である．インスリン抵抗性改善薬pioglitazoneは，食事療法，運動療法のみでは十分な効果がえられず，インスリン抵抗性があるか，もしくはスルホニル尿素薬で血糖降下が不十分な2型糖尿病に適応がある[8]．そこで糖尿病を合併したDM1のインスリン抵抗性を改善し，血糖をコントロールすることを目的にpioglitazone内服治療の効果を検討した．

対象と方法

当院に入院中で，末梢血白血球でミオトニンプロテインキナーゼ遺伝子の非翻訳領域のCTGリピート延長があるDM1患者で，糖尿病を合併した8人（男性6人，女性2人），平均年齢52.1±8.6歳（32～60歳）を対象とした（Table 1）．同一家系の者はいなかった．患者の移動能力は2人（患者2，3）は歩行器で自力歩行可能で，他の6人は車椅子で自力移動できた．Body mass index（BMI）[8]では患者3，6の2人がBMI 25以上で肥満であった．日本糖尿病学会の診断基準に準拠し[8]，(1) 随時血糖200mg/dl以上を確認，(2) 早朝空腹時血糖126mg/dl以上を確認，(3) 75g糖負荷試験（75gOGTT）で120分後の血糖200mg/dl以上を確認，のいずれかで糖尿病型とし，別の日にふたたび糖尿病型が確認されたものを糖尿病と診断した．患者1，3，5，6は診断基準（1）を満たし，患者2，4，7，8は（1）と（3）を満たした．（2）を満たす患者はいなかった．患者1～5は血糖降下薬による治療をおこなっておらず，食事制限のみおこなっていた．患者6～8はglibenclamide（Euglucon®）を規則的に内服していたが，血糖のコントロールは不良で，月に1～2回程度，空腹時に低血糖症状が生じることがあった．そのため3人ともglibenclamideを減量した既往があり，患者7は2.5mgから最終的には0.25mgまでglibenclamideを減量していた．いずれの患者もglibenclamideの内服を中止すると高血糖が生じた．Homeostasis model assessment-insulin resistance（HOMA-IR）は末梢のインスリン抵抗性の評価指数で，空腹時血糖（mg/dl）×空腹時インスリン値（μU/ml）/405から算出した[9]．胸部X線写真，心電図，ヒト心房性ナトリウム利尿ペプチド（HANP）は全例が正常範囲にあり，心不全を示唆するものはなかった．

患者1～5は糖尿病の診断後，口頭による説明と同意のもとにpioglitazone（Actos®）15mgを朝食後に内服した．患者6～8は，glibenclamide内服治療で血糖のコントロールが困難であることを説明し，同意をえてglibenclamideを中止し，朝食後pioglitazone 15mg内服に変更した．内服開始前1カ月以内の検査値と治療中の最新の検査値を比較し治療効果を判定し

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Table 1 Pretreatment profile of patients

Patient	Age(year)/ Sex	CTG repeat	Walking ability	BMI (kg/m²)	Dose of glibenclamide (mg)	Casual plasma glucose (mg/dl)	HbA_{1c} (%)	HOMA-IR
1	51/M	400	WC	17.8	—	230	6.1	0.6
2	60/F	1,700	WA	18.2	—	289	6.1	2.3
3	52/M	1,400	WA	25.0	—	202	5.7	2.8
4	54/F	900	WC	16.0	—	269	6.8	1.6
5	54/M	1,000	WC	20.6	—	225	5.6	2.7
6	32/M	1,400	WC	26.9	1.25	213	7.9	2.8
7	57/M	1,100	WC	21.6	0.25	257	6.3	0.6
8	57/M	1,400	WC	17.1	1.25	231	7.0	3.3
Mean ± SD	52.1 ± 8.6	—	—	20.4 ± 3.9	—	239.5 ± 29.5	6.4 ± 0.8	2.1 ± 1.0

BMI : body mass index, HOMA-IR : homeostasis model assessment-insulin resistance, M : male, F : female, WC : wheelchair, and WA : walking with aid.

た．なお，患者6～8はglibenclamide内服中にpioglitazone内服開始前の検査をおこなった．75gOGTTでは糖負荷前，糖負荷30分後，60分後，90分後，120分後，180分後に血糖とIRIを測定した．治療効果の評価には75gOGTTでの糖負荷後120分の血糖と糖負荷後120分までのIRI曲線下面積（ΣIRI(μU・hr/ml)），HOMA-IRを比較した．治療期間中はBMI，HbA_{1c}を測定し，HANPをふくめた血液検査，胸部X線写真，心電図，心エコー，眼科検診を定期的におこなった．解析にはStatView (ver. 5.0)®を使用し，paired t検定でいずれも$p<0.05$を有意とした．

結　果

pioglitazone治療は平均14.8±9.1カ月間（6～36カ月）観察した．観察期間中に脱落した患者はいなかった．

75gOGTTの変化（Fig. 1）：糖負荷前の血糖は全例で126 mg/dl以下であった．血糖降下薬の内服歴がない患者5人のうち3人は，治療前の75gOGTTで一相性に血糖が上昇するパターンであり，患者1は糖負荷90分後に，患者2は糖負荷60分後に，患者3は糖負荷30分後に，それぞれ血糖のピークがあった(Fig. 1-A)．患者4，5は，糖負荷180分後にふたたび血糖が上昇する二相性パターンであった（Fig. 1-B)．IRIのパターンは個人差があり，IRIのピークの時間は一定しなかった．pioglitazone治療中のIRIの変化も個人差があった．glibenclamideからpioglitazoneに内服薬を変更した患者6，7，8は，75gOGTTの血糖はわずかに低下した．しかし，患者6，7ではすべての測定で治療前よりも治療中のIRIが低下した（Fig. 1-C)．

糖負荷120分値，ΣIRI，HOMA-IR（Fig. 2）：pioglitazone治療前にくらべて治療中は全例で糖負荷後120分の血糖が低下し，治療前の平均203.3±41.7mg/dlから治療中は平均153.9±39.5mg/dlに有意に改善した（$p=0.004$）(Fig. 2-A)．ΣIRIは，pioglitazone治療前の平均236.9±170.2μU・hr/mlから治療中は平均169.6±81.3μU・hr/mlに低下傾向をみとめた（$p=0.12$)．治療前にΣIRIが250μU・hr/ml以上であった4人は治療中にΣIRIが減少し，治療前にΣIRIが150μU・hr/ml以下であった4人は治療中にΣIRIは軽度増加した(Fig. 2-B)．HOMA-IRは治療前の平均2.1±1.0から治療中は1.1±0.4に有意に改善した（$p=0.04$)．治療前にHOMA-IRが2.5以上であった4人は，治療中はHOMA-IRが下がった．また，8人中7人は治療中のHOMA-IRが1.5以下であった(Fig. 2-C)．

BMI，HbA_{1c}，副作用（Table 2）：Pioglitazone治療前と治療中でBMIとHbA_{1c}に有意な変化はなかった（$p=0.34$，$p=0.21$)．治療期間中に新たな肝機能障害，腎機能障害，心不全，低血糖発作，網膜症は生じなかった．患者8はHANPが軽度に上昇したが，胸部X線写真，心エコーで心機能低下はなく，pioglitazone内服治療を継続した．

考　察

DM1患者は腹部症状などから欠食することも多く，食事量が一定せず，スルホニル尿素薬のようなインスリン分泌を促進する薬剤[8]の内服やインスリン皮下注射による糖尿病の治療では低血糖を生じる危険がある．また，DM1では空腹時に糖を負荷すると急激に過剰なインスリンが分泌され，低血糖発作が誘発されることも報告されている[10]．glibenclamideからpioglitazoneに変更した患者もふくめ，pioglitazone治療中は低血糖発作がおきず，臨床的に有用な治療と考えた．

75gOGTTでのDM1の血糖の変動は，正常型，糖尿病型，二相性など，多様であることが報告されている[11][12]．本研究の対象患者でも糖負荷後の血糖の上昇パターンは様々であったが，いずれのパターンであってもpioglitazone治療中の血糖は改善した．ΣIRIの変動は個人差があった．ΣIRIが250μU・hr/ml以上の過分泌の患者はΣIRIが減少し，インスリン必要量が減少して，血糖も改善した．ΣIRIが150μU・hr/ml以下の分泌不良患者は，ΣIRIは軽度増加した．pioglitazone内服によってDM1のインスリン分泌量が良好に調節された可能性がある．また，HOMA-IRは一般に2.5以上でインスリン抵抗性があり，1.5以下で正常とされている[8]．この基準に

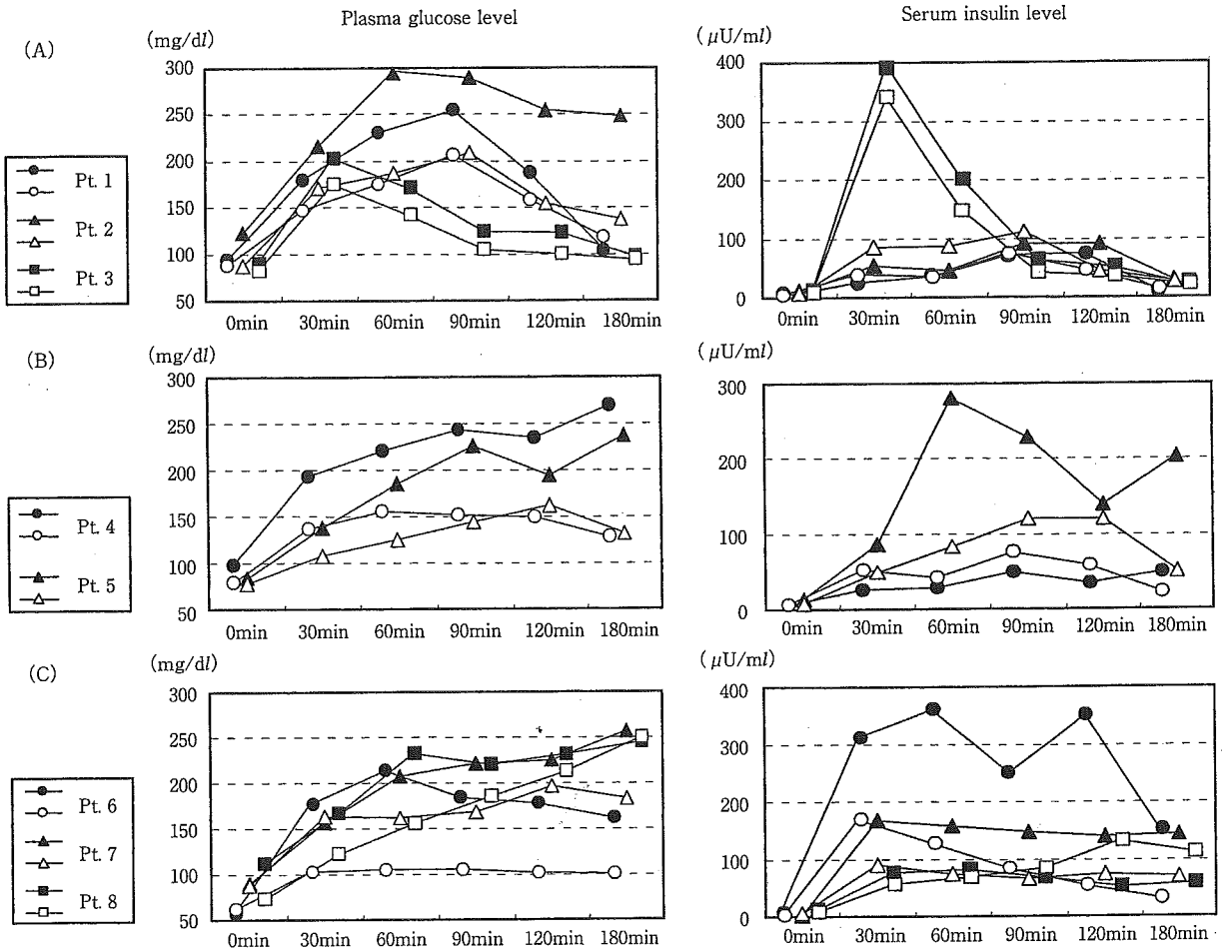

Fig. 1 Results of 75g oral glucose tolerance test. Patient 1, 2, and 3 show monophasic increase in plasma glucose (A), Patient 4 and 5, biphasic increase in plasma glucose 180 min later (B). Patient 7, 8, and 9 are under glibenclamide treatment (C). Plasma insulin levels decrease significantly in patient 5, 6 and 7 but remain unchanged in others. Pt.: patient.●,■,▲: pretreatment and○,□,△: during pioglitazone treatment.

よると，本研究の8人中4人はpioglitazone治療前にインスリン抵抗性があったが，治療中はインスリン抵抗性はみとめられなかった．pioglitazoneはDM1のインスリン抵抗性を改善したと考えた．とくにインスリン過分泌の糖尿病の患者で有用性が高い可能性がある．

これまでにDM1にインスリン抵抗性を改善するとされるチアゾリジン誘導体の一つであるtroglitazoneを投与した報告がある．糖尿病を合併したDM1の22歳女性では，troglitazoneを12週間投与したところ，インスリン抵抗性が改善し，臨床的にはミオトニアが改善した[13]．しかし，糖尿病にはいたらないDM1の35歳男性では，amitriptylineに併用してtroglitazoneを6週間投与したところ，インスリン基礎値が上昇し，インスリン過分泌反応が増悪した．そして，troglitazone中止4週後の検査ではインスリン過分泌が改善したため，troglitazoneはインスリン抵抗性を改善させないと考察された[14]．troglitazoneは重篤な肝機能障害の副作用のため製造中止になり，結局DM1に合併した糖尿病への治療効果の評価は定まらなかった．しかしpioglitazoneではインスリン抵抗性は改善され，インスリン過分泌は抑制された．なおミオトニア現象に対する効果は，対象患者では明らかではなかった．

pioglitazoneの有害事象は，肝機能障害は少なく，体液貯留による心不全が報告されている[15]．本研究の患者では，8人中1人にHANPの上昇がみられたが，心不全ではなかった．DM1では心不全がなくても脳性ナトリウム利尿ペプチド(BNP)が上昇することは多く，心機能の評価にはHANPがより有用であることが示唆されている[16]．しかし，心機能が正常でもHANPが上昇するという報告[17]もあり，患者のHANP上昇の意味づけは困難であった．pioglitazone治療中は胸部X線写真などでの経過観察は必要と考えるが，DM1ではpioglitazone内服で心不全を発生する危険がとくに高いとは考えにくい．

一般に2型糖尿病発症の経過は，まずインスリン抵抗性の獲得によって糖利用が障害され，血糖を保つために膵β細胞

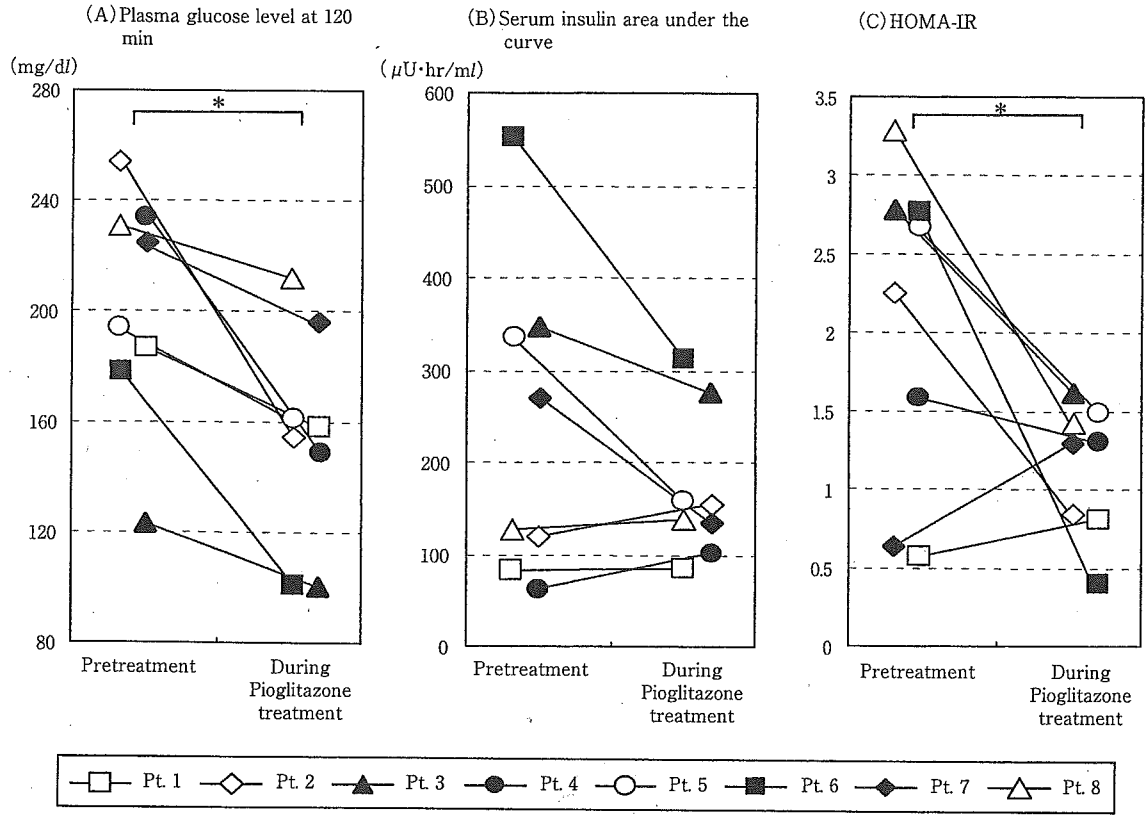

Fig. 2　Plasma glucose level at 120 min of 75g oral glucose tolerance test (75gOGTT) (A), serum insulin area under the curve (ΣIRI) of 75gOGTT (B) and the homeostasis model assessment-insulin resistance (HOMA-IR) (C). Plasma glucose level at 120 min decreased during pioglitazone treatment in all patients. ΣIRI decreased patient 3, 5, 6 and 7 with a pretreatment Σ IRI≧250μU・hr/ml, while it increased in other patients with pretreatment Σ IRI≦150μU・hr/ml slightly. HOMA-IR improved significantly during pioglitazone treatment in patient 3, 5, 6 and 8. Pt.: patient.* : $p<0.05$.

Table 2　During pioglitazone treatment.

Patient	Pioglitazone administration period (months)	BMI (kg/m^2)	HbA1c (%)	HANP (pg/ml)
1	12	17.8	6.0	< 10
2	12	19.1	6.4	25
3	9	25.0	5.6	16
4	16	15.7	6.5	14
5	12	19.8	5.4	< 10
6	36	25.7	4.6	10
7	6	21.6	6.5	26
8	15	16.5	6.1	47
Mean ± SD	14.8 ± 9.1	20.2 ± 3.7	5.9 ± 0.7	—

BMI : body mass index, HANP : human atrial natriuretic peptide.

からのインスリン分泌が促進する．やがて膵β細胞の疲弊によってインスリン分泌が低下し，高インスリン血症でありながら血糖が上昇する相対的インスリン不足の状態になり，さらに絶対的インスリン分泌不足による糖尿病へと進行すると考えられている[18]．もともとインスリン抵抗性があり，高インスリン血症をともなうDM1患者が2型糖尿病と類似の経過をたどるとすると，pioglitazone治療によってDM1の膵β細胞の疲弊を予防することで，糖尿病の増悪を阻止する効果が期待できる可能性がある．しかし，現時点では，HbA_{1c}が正常なDM1患者にpioglitazone治療をおこなう必要性や耐糖能障害を改善することでどのような合併症を予防でき，それが生命予後とどのようにかかわるのかなど，不明な点は多い．

今後，さらに長期にわたって pioglitazone 治療を経過観察するとともに，糖尿病を発症する前に pioglitazone を投与し，膵β細胞の疲弊を予防することで糖尿病の発症を阻止できるかを検討すべきであろう．

高インスリン血症（ΣIRI＞90μU・hr/ml），肥満（BMI≧25 kg/m²），高血圧，脂質代謝異常，動脈硬化性病変などの因子は，それぞれが軽度であっても同時に存在することで虚血性心疾患などの発症リスクを上げるとされ，インスリン抵抗性症候群という概念が提唱されている[19]．DM1 ではインスリン抵抗性と脂質代謝異常をみとめ，内臓脂肪も多く[20]，動脈硬化がいちじるしいことも少なくない．pioglitazone には内臓脂肪を減少させる効果もあり[21]，pioglitazone 投与によって DM1 患者の持つ複数の危険因子を減らせる可能性がある．しかし，DM1 患者で虚血性心疾患が多いか，また DM1 でも危険因子として重要かどうかは不明であり，検討すべき課題と考える．DM1 の標準的な医療として確立するためには，より多くの DM1 患者で pioglitazone 治療の長期経過を観察する必要がある．

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