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多発性硬化症
Multiple sclerosis

Key point

◉多発性硬化症（MS）は代表的な自己免疫疾患であり，自己反応性Th1細胞が病態に深く関与している．
◉IFN－γ，IL－12，IL－23などTh1細胞を活性化するサイトカインは病態を増悪させ，IL－4，IL－10，IL－13などのTh2サイトカインは病態を改善させる方向に作用すると考えられる．
◉しかし，すべての症例においてTh1制御が有効であるか確証はない．
◉なお，関節リウマチと異なりMSにはTNF－α阻害剤は無効である．

多発性硬化症（multiple sclerosis：MS）は，大脳，脊髄，視神経などに炎症性脱髄病変を多発する疾患であり，代表的な中枢神経系の炎症性疾患である[1]．これまで日本では視神経と脊髄に病変の限局する症例が多いといわれてきた．しかし，近年大脳に病変の多発する“欧米型MS”が80％以上を占めるようになってきており，すくなくとも若年者については欧米のMSと大きな違いがないようである．病因についてはミエリン塩基性蛋白（myelin basic protein：MBP）などの自己抗原に対する免疫反応（T細胞およびB細胞）が関与する自己免疫疾患であり，とくにTh1細胞が脳内炎症の引き金を引く重要な細胞成分であると考えられている．一卵性双生児の研究などから発症には遺伝因子と環境因子の双方が複雑に作用すると考えられている．治療法としては急性期にはステロイドパルス療法を行うが長期的な予後を改善する治療として最近では慢性期のインターフェロンベータ（IFN－β）自己注射が推奨されている．

MSの病態

実験的自己免疫性脳脊髄炎（experimental autoimmune encephalomyelitis：EAE →サイドメモ）の研究からMSにおいても中枢神経髄鞘を標的とするTh1細胞の役割が推測されてきた．患者末梢血T細胞を利用した研究結果や治療薬に対する反応性の解析結果はこの仮説を支持している．

Th1細胞は病原体を貪食したマクロファージの活性化に必須であり，IFN－γ，IL－2などのTh1サイトカインを産生する．MSの病変部位で活性化CD4陽性T細胞が検出できること，MS治療薬コポリマーⅠの投与によってTh2シフトが起こることなど，MSがTh1細胞を介する病気であることを支持する知見が数多くみられる．また，治療効果が期待されたMBPアナログの投与が一部の症例で激しい再発を誘導した事実も，MSにおけるTh1自己反応性T細胞の重要性を示唆する[2]．しかし，病理所見で補体の沈着が顕著な症例，CD8陽性細胞がめだつ症例，血漿交換が有効な症例もあり，“MSはTh1病”という単純なとらえ方には無理がある．脳内の炎症を惹起するには脳血液関門が破綻する必要があり，Th1細胞はその最初のプロセスに重要な役割を果たす．しかし，ひとたび炎症がはじまれば抗体やマクロファージがさまざまなレベルで関与すると考えられる．

MS病態に関与するサイトカイン

他稿でも詳述されているが，Th細胞はサイトカイン産生パターンによってTh1細胞とTh2細胞に区別される．Th1細胞は，IFN－γやIL－2，Th2細胞はIL－4，IL－5，IL－10，IL－13などをおもに産生する．IL－12はTh1細胞の誘導を促進し，IL－4はTh2細胞への分化に重要である．Th1細胞とTh2細胞は産生サイトカインによってたがいに制御しあっており，健常時にはTh1とTh2の平衡が保たれている．Th1/Th2バランスという観点でMSの病態を考えた場合，IFN－γ，IL－2，IL－3，IL－12，IL－18などTh1細胞の働きを補助するサイトカインは，MSの病態を増悪させる方向に働き，IL－4，IL－5，IL－6，IL－10，IL－13，IL－21などのTh2サイトカインは脳炎惹起性Th1細

サイドメモ　実験的自己免疫性脳脊髄炎（EAE）

中枢神経系の炎症病変に起因する麻痺症状を引き起こすMSの動物モデルである．ミエリン塩基性蛋白やMOGなどの髄鞘蛋白を感作抗原としてアジュバントとともに接種することによって誘導される．感作後10日前後で尻尾の麻痺が現れ，その後不安定な歩行，下肢麻痺，上肢麻痺と症状が進行するが，自然に回復することが多い．抗原特異的なTh1細胞が炎症の引き金を引き，Th2偏倚を誘導するような操作によって症状が軽快する．

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胞を制御する方向に働くものと理解される．一方，治療薬として処方されているIFN－βには脳血液関門を安定化させてMSの再発を低下させる効果がある．以下にMSの病態に関与するおもなサイトカインについて論じる．

1．IFN－γ

T細胞依存性のマクロファージ活性化の中枢を担う代表的なTh1サイトカインであり，抗原刺激されたTh1細胞の生成を促進しTh2細胞を抑制する．MS患者の血液，髄液中での増加が確認されており，病態の進展に関与すると考えられる．サイトカインの機能に関する情報の乏しい1980年代に，IFN－γを患者に投与する臨床試験が行われた．しかし，病状の悪化する患者が多く中止された[3]．この不幸な臨床治験の結果はMSがTh1細胞を介する病気であることを裏書きする事実として，しばしば引用されている．

2．IFN－β

MSがウイルス性疾患であると信じられた時代に，その抗ウイルス作用を期待されてIFN－βの治験が行われた．その結果，再発回数の減少，MRIで描出される病変数の低下などの効果が確認され，MSの病態を修復する薬剤（disease modifying agent）として認可された．現在では内外で広く処方されているが，その作用機構については諸説ある．Th1/Th2バランスのTh2偏倚を強調する論文もあるが，著者らのDNAマイクロアレイを使った研究では確認できなかった[4]．むしろ，IFN－βによる抗炎症性蛋白の誘導やケモカインの抑制によって治療効果の一部が説明できるかもしれない．

3．TNF－α

TNF－αは，IFN－γと同様にMS患者の血液，髄液中において増加している炎症性サイトカインである．TNF－αを過剰発現させたマウスではマクロファージ浸潤やグリオーシスを伴った脱髄病変を自然発症し，またEAEを誘導すると通常マウスよりも重症化する．培養細胞の実験ではTNF－αがオリゴデンドロサイトを傷害することが明らかになっており，MSにおいても脱髄病変形成に関与していることが推測される．しかし，TNF－αの中和抗体や受容体阻害剤は，MSを抑制しないばかりか，MS発症の誘因になることが示唆されている．その理由はまだ明らかになっていない．

4．Transforming growth factor（TGF）－β

細胞増殖や分化の抑制に働くサイトカインであり，MS脳内病変ではアストロサイトに強く発現している．回復期の炎症終結に関与していると考えられている．また，抗原の経口免疫寛容を担う細胞をTh3細胞とよび，その細胞が産生するTGF－βが重要な役割を果たしているという報告もある．

5．IL－4

Th2の増殖や分化を促進する重要なサイトカインで，その結果，IL－4やIL－5の産生を促す．また，マクロファージの活性化を抑えてIFN－γ産生を抑制し，IFN－γの作用自体に拮抗してTh1免疫応答を阻害する．MSではコポリマーI投与後に抗原特異的T細胞のIL－4産生亢進が確認されている．MSの寛解期ではCD4陽性のNKT細胞においてIL－4産生能が亢進している[5]．IL－4はMSの寛解維持に関与するサイトカインと考えられる．

6．IL－5

MS寛解期の患者から分離したNK細胞では健常人のものと比べてCD95弱陽性細胞の比率が増加しており，IL－5 mRNA発現レベルが著明に上昇していることがわかっている．そのNK細胞をPMA/ionomycinで刺激すると培養上清中のIL－5濃度は健常人群に比べて有意に亢進している[6]．このような性格をもつNK細胞はNK2細胞と分類されるようになってきたが，MS再発時にはNK2の傾向は消失している．NK2細胞にはTh1細胞の誘導を抑制する働きがあり，抗IL－5抗体でその働きが中和される．以上より，MS寛解維持にはNK細胞がNK2の働きを示してTh1を抑制しており，この抑制にIL－5が重要な役割を果たしているものと推測される．

7．IL－10

IL－4と同様，Th2細胞が産生するサイトカインでTh1細胞のIFN－γ産生を抑制する．MS病変では血管周囲のマクロファージで強く発現しており，病変の炎症終結に重要な役割を果たしていると考えられている．IL－10欠損マウスではEAEが重症化する．また，低容量抗原を用いた経粘膜免疫寛容ではIL－10が重要な役割を果たす．このように，Th1細胞の関与する自己免疫疾患においてIL－10は重要な制御因子である．

8．IL－12

抗原提示細胞由来のIL－12はTh1への分化に必須であり，MSの病態に深く関与していると考えられる．活動期MSの病変部位ではIL－12が強く発現しており，髄液中IL－12も上昇している．IL－12はp35とp40のヘテロダイマーとしてはじめて生理活性を示す．機能的IL－12は浸潤マクロファージや活性化ミクログリアから産生されていると考えられている[7]．

9．IL－18

IL－18は多様な細胞から産生され，おもにTh1サイトカイン産生に関与する．IL－18単独ではT細胞

やNK細胞に少量のIFN－γを産生させるのみであるが，IL－12の存在下では大量のIFN－γ産生を促す．EAE誘導時にIL－18を投与するとEAE症状は悪化する．逆にIL－18に対する抗体を投与すると，IFN－γやTNF－αの産生抑制とIL－4の産生亢進を伴ってEAEは抑制される[8]．MSでは末梢白血球のmRNAレベルにおいてIL－18が増加しており，とくに二次進行型の症例において顕著であった．また，血清や髄液中のIL－18もMS患者で増加しているという報告もある．

10．IL－23

IL－23はIL－12のファミリーであり，メモリーT細胞，マクロファージ，樹状細胞などから産生される．IL－12とはすこし異なり，IL－23はp19とp40で構成されている．これらサブユニットのノックアウトマウスを用いたEAEの実験ではp19KOではTh1細胞の分化やIFN－γ産生が正常であるにもかかわらずEAEがまったく誘導されなかった．しかし，p19KOにEAE感作8日目にIL－23を投与するとEAEは発症し，IL－12の投与ではまったく発症しなかった．一方，p40KOではEAEはまったく生じず，IFN－γ産生は低下していた．これらの結果からIL－23はEAEにおいて重要であり，とくに中後期の炎症維持に必要であると考えられる[9]．MSにおいてもIL－23をターゲットにした治療法が今後期待される．

MS治療とTh2サイトカイン

脳炎惹起性Th1細胞のかかわる病態を是正するという観点から，種々のMS治療の試みがなされている．IL－4を直接投与する方法はEAEでは有効であったが，MS患者では副作用の問題から失敗に終わった．また，自己抗原を投与し免疫寛容を誘導するという方法はEAEではIL－10やIL－13などTh2サイトカイン産生が促され，治療効果をもたらした．Weinerらはbystander suppressionという抗原非特異的な抑制効果を期待して，MSに中枢神経ミエリンを経口投与したが，有意な治療効果は証明できなかった．

著者らが開発した新規糖脂質リガンドによる治療はnatural killer T（NKT）細胞を介して選択的にTh2サイトカインであるIL－4を産生させるという方法である．EAEの誘導時にその糖脂質（OCHと命名）を経口投与するとTh1/Th2バランスが是正されEAEは有意に軽症化した[10]．幸いマウスとヒトのNKT細胞は同じリガンドに反応するため，このEAEの成果がMS治療に応用できる可能性が期待できる．

おわりに

本稿で述べたように，MSの病態にはさまざまなサイトカインが関与している．従来MSはTh1病であるという考え方が主流で，Th1サイトカインは悪玉で，Th2サイトカインは善玉とされてきた．しかし，生体内での作用は複雑であり，同じサイトカインが状況によっては増悪にも保護的にも作用しうる可能性があり，また抑制的に働くはずのTh2免疫応答が症例や病期によっては有害であることも報告されている．Th1細胞がおもに関与するようなEAE類似の病態と抗体の関与の大きい病態を切り離して議論する必要が生じている．すなわち，MSの免疫分子論的な亜分類が今後の研究の焦点のひとつになっている．また，サイトカインとMSの病態を考えるとき，単にTh1/Th2バランスという観点だけではなく，Th細胞以外の免疫系細胞も含めた病態との相関関係を考慮しながらサイトカインの解析を進めていく必要がある．一度に多数の分子発現を解析するDNAマイクロアレイなどの導入によって，これまでに注目されていないサイトカイン，またはサイトカイン様物質の重要性が今後明らかになる可能性も十分にある．

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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) K□□□□□□□□□□. 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.

t1.1 Table 1
t1.2 Demographic characteristics of the study populations

t1.3 Characteristics	Multiple sclerosis (MS) patients	Healthy control (CN) subjects
t1.4 The number of the study population (*n*)	72	22
t1.5 Age (average ± SD, year)	36.1 ± 10.3	38.6 ± 12.3
t1.6 Sex (male vs. female)	17 vs. 55	6 vs. 16
t1.7 Disease course (RRMS vs. SPMS)	65 vs. 7	(–)
t1.8 Disease subtype (conventional MS vs. non-conventional MS)	57 vs. 15	(–)
t1.9 Disease duration (average ± SD, year)	7.7 ± 5.4	(–)
t1.10 EDSS score (average ± SD, score)	2.8 ± 2.0	(–)
t1.11 Number of lesions on T2-weighted MRI (average ± SD, number)	24.7 ± 31.9	(–)
t1.12 Number of relapses during 2 years before blood sampling (average ± SD, number)	1.9 ± 1.5	(–)
t1.13 Day of IVMP treatment during 2 years before blood sampling (average ± SD, day)	5.9 ± 5.8	(–)
t1.14 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 nonconventional MS that affects chiefly the optic nerve and the spinal cord. Abbreviations: RRMS, relapsing–remitting MS; SPMS, secondary progressive
t1.15 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 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.

t3.1 Table 3

t3.2 Top 30 genes expressed differentially in non-T cells between MS and controls

t3.3	No.	Symbol	GenBank accession number	Description	Presumed function	Possible involvement in apoptosis regulation	Significance (p-log)
t3.4	*The upregulated genes*						
t3.5	1	ICAM1	NM_000201	Intercellular adhesion molecule-1	a cell surface glycoprotein ligand (CD54) for LFA-1 and Mac-1	(+)	1.11E−09
t3.6	2	CDC42	NM_001791	Cell division cycle 42	a small GTPase that regulates diverse cellular functions	(+)	1.49E−08
t3.7	3	RIPK2	NM_003821	Receptor-interacting serine/threonine kinase 2	a protein kinase interacting with CLARP in the Fas-signaling pathway	(+)	1.88E−07
t3.8	4	IL1R2	NM_004633	IL-1 receptor, type II	a decoy receptor for IL-1 that inhibits IL-1 activity	unknown	4.56E−07
t3.9	5	MAD	NM_002357	Max dimerization protein	a transcriptional repressor that competes with MYC for binding to MAX	(+)	1.00E−06
t3.10	6	CXCL2	NM_002089	Chemokine, CXC motif, ligand 2	a chemokine designated MIP2 binding to CXCR2	(+)	1.91E−06
t3.11	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
t3.12	8	TOP2A	NM_001067	Topoisomerase 2 alpha	a DNA topoisomerase	(+)	4.82E−06
t3.13							
t3.14	*The downregulated genes*						
t3.15	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
t3.16	10	RGS14	NM_006480	Regulator of G protein signaling 14	a downregulator of signaling through G protein-coupled receptors	(+)	5.44E−08
t3.17	11	COX15	NM_078470	Cytochrome c oxidase assembly protein COX15	a protein essential for assembly of COX	(+)	6.43E−08
t3.18	12	AKAP11	NM_016248	A-kinase anchor protein 11	a protein anchoring the regulatory subunit of protein kinase A	(+)	1.68E−07
t3.19	13	TCF17	NM_005649	Transcription factor 17	a transcriptional repressor of renal genes designated Kid-1	(+)	1.92E−07
t3.20	14	CDC25B	NM_021874	Cell division cycle 25B	a tyrosine phosphatase that activates the cyclin dependent kinase CDC2	(+)	2.40E−07
t3.21	15	GZMA	NM_006144	Granzyme A	a cytotoxic T cell- and NK cell-specific serine protease	(+)	2.49E−07
t3.22	16	CHST4	NM_005769	Carbohydrate sulfotransferase 4	an *N*-acetylglucosamine 6-O sulfotransferase	unknown	3.46E−06
t3.23	17	BCL2	NM_000633	B-cell CLL/lymphoma 2	a mitochondrial membrane protein that blocks the apoptotic death	(+)	4.81E−07
t3.24	18	CR2	NM_001877	Complement component receptor 2	a membrane receptor (CD21) for C3d	(+)	5.88E−07
t3.25	19	RPA1	NM_002945	Replication protein A1	a single-stranded DNA-binding protein that regulates DNA replication	(+)	6.72E−07
t3.26	20	POLR2H	NM_006232	RNA polymerase II, subunit H	a subunit of RNA polymerase II	(+)	7.28E−07
t3.27	21	E2F5	NM_001951	E2F transcription factor 5	a transcription factor of the E2F family	(+)	1.00E−06
t3.28	22	RAB7L1	NM_003929	Ras associated protein RAB7-like 1	a RAS-related small GTP-binding protein	(+)	1.49E−06
t3.29	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
t3.30	24	HSPA1L	NM_005527	Heat shock 70-kD protein-like 1	a constitutive member of the HSP70 family	(+)	1.87E−06
t3.31	25	RBBP4	NM_005610	Retinoblastoma-binding protein 4	a nuclear protein binding to RB1	(+)	3.13E−06
t3.32	26	PRKDC	NM_006904	Protein kinase, DNA-activated, catalytic subunit	a nuclear serine/threonine protein kinase	(+)	3.36E−06
t3.33	27	RASSF1	NM_170714	Ras association domain family 1	a lung tumor suppressor gene having a Ras-association domain	(+)	3.49E−06
t3.34	28	DAXX	NM_001350	Death-associated protein 6	a protein that interacts with the death domain of Fas	(+)	5.16E−06
t3.35	29	EGF	NM_001963	Epidermal growth factor	a potent mitogenic factor for the cells of both ectodermal and mesodermal origin	(+)	5.74E−06
t3.36	30	NPR2L	NM_006545	Nitrogen permease regulator 2-like	a possible tumor suppressor gene	unknown	1.13E−05

t3.37 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.

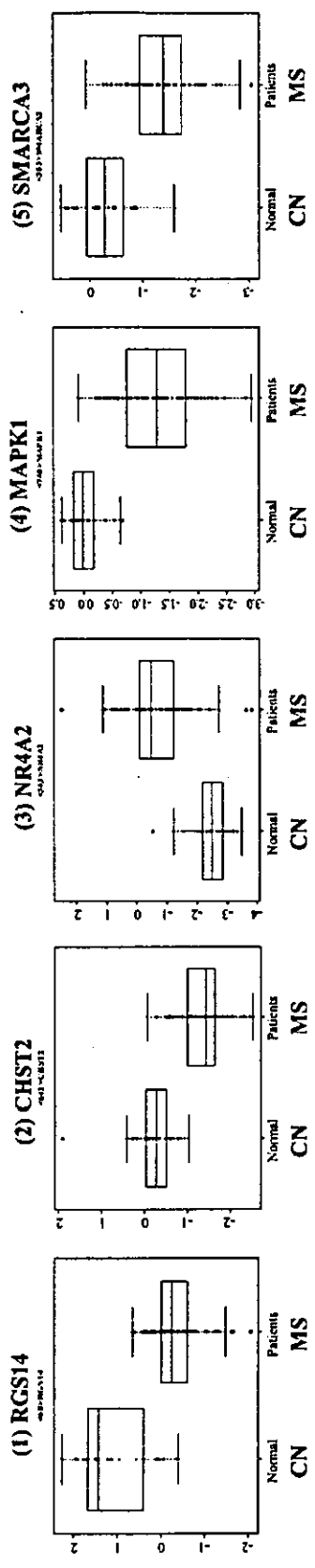

Fig. 1. Top 10 differentially expressed genes in T cell fraction between MS and CN groups. The gene expression profile was studied in $CD3^+$ T cell fraction isolated from 72 MS patients and 22 healthy control (CN) subjects by analyzing a cDNA microarray containing 1258 genes. RNA of MS and CN samples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

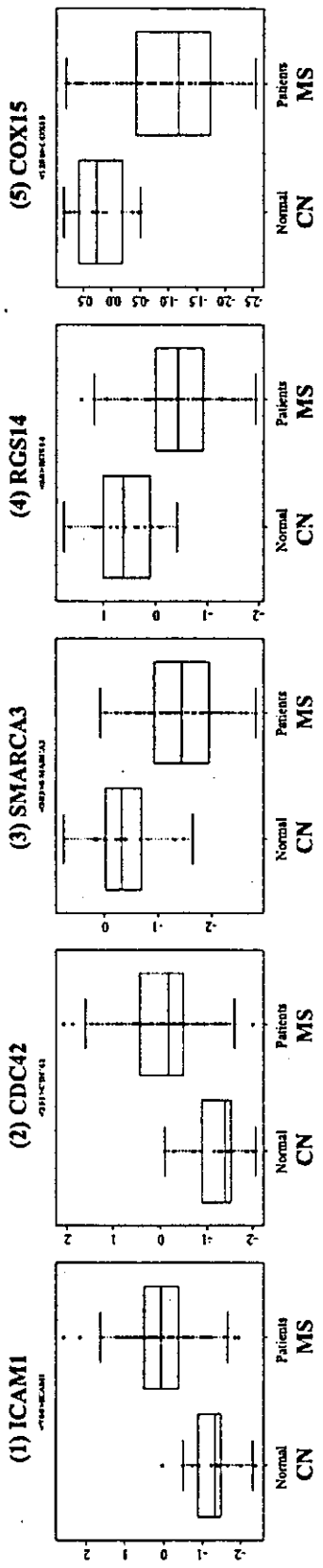

Fig. 2. Top 10 differentially expressed genes in non-T cell fraction between MS and CN groups. The gene expression profile was studied in $CD3^-$ non-T cell fraction isolated from 72 MS patients and 22 CN subjects by analyzing 1258 cDNA microarrays. See Fig. 1. Top 10 genes are shown as box and whisker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

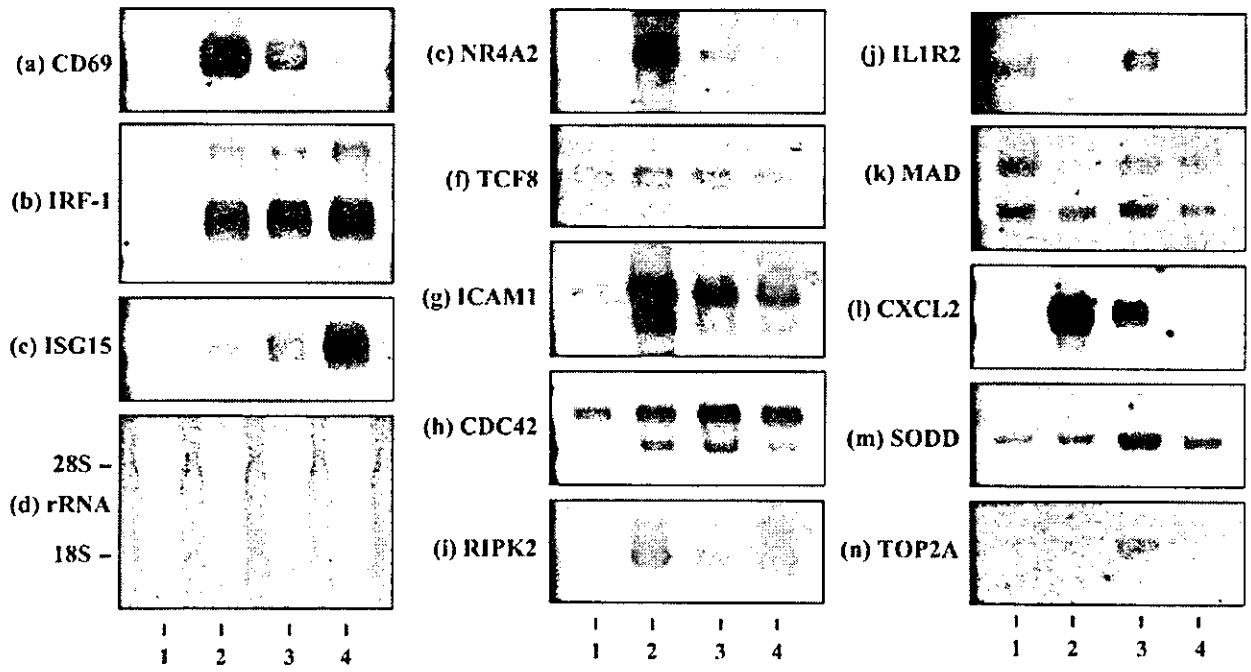

Fig. 3. The genes upregulated in MS were expressed in cultured PBMC in an activation-dependent manner. Unfractionated PBMCs of a healthy subject were incubated for 6 h in medium without (lane 1) or with inclusion of 25 ng/ml PMA and 1 μg/ml IOM (lane 2), or for 24 h in the plate coated with 1 μg/ml anti-CD3 mAb (lane 3) or in the medium containing 100 ng/ml IFNγ□□□□□. 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.

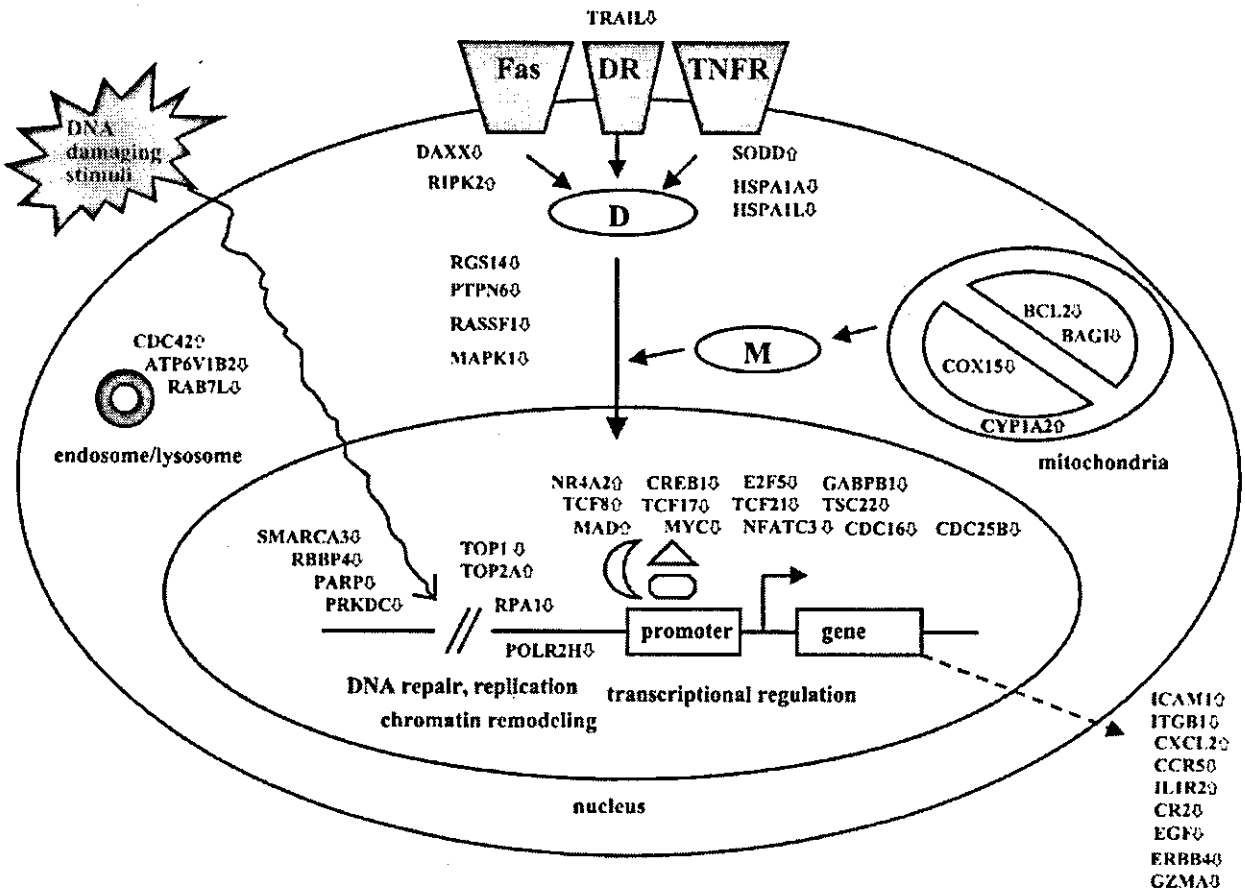

Fig. 4. Aberrant expression of apoptosis signaling-related genes in MS lymphocytes. More than 80% of the top 30 differentially expressed genes between MS and CN groups were categorized into apoptosis signaling-related genes of both proapoptotic and antiapoptotic classes, whose expression was either upregulated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

Uncited reference

Schwab et al., 1998

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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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The 14-3-3 Protein ε Isoform Expressed in Reactive Astrocytes in Demyelinating Lesions of Multiple Sclerosis Binds to Vimentin and Glial Fibrillary Acidic Protein in Cultured Human Astrocytes

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The 14-3-3 protein family consists of acidic 30-kd proteins expressed at high levels in neurons of the central nervous system. Seven isoforms form a dimeric complex that acts as a molecular chaperone that interacts with key signaling components. Recent studies indicated that the 14-3-3 protein identified in the cerebrospinal fluid of various neurological diseases including multiple sclerosis (MS) is a marker for extensive brain destruction. However, it remains unknown whether the 14-3-3 protein plays an active role in the pathological process of MS. To investigate the differential expression of seven 14-3-3 isoforms in MS lesions, brain tissues of four progressive cases were immunolabeled with a panel of isoform-specific antibodies. Reactive astrocytes in chronic demyelinating lesions intensely expressed β, ε, ζ, η, and σ isoforms, among which the ε isoform is a highly specific marker for reactive astrocytes. Furthermore, protein overlay, mass spectrometry, immunoprecipitation, and double-immunolabeling analysis showed that the 14-3-3 protein interacts with both vimentin and glial fibrillary acidic protein in cultured human astrocytes. These results suggest that the 14-3-3 protein plays an organizing role in the intermediate filament network in reactive astrocytes at the site of demyelinating lesions in MS. *(Am J Pathol 2004, 165:577–592)*

The 14-3-3 protein family consists of evolutionarily conserved, acidic 30-kd proteins originally identified by two dimensional analysis of brain protein extract.[1–4] Seven isoforms of the 14-3-3 protein named β, γ, ε, ζ, η, θ (also termed as τ), and σ have been identified in eukaryotic cells. Although the 14-3-3 protein is widely distributed in neural and nonneural tissues, it is expressed most abundantly in neurons in the central nervous system (CNS), where it represents 1% of total cytosolic proteins.[4–7] A homodimeric or heterodimeric complex, which is composed of the same or distinct isoforms of the 14-3-3 protein, constitutes a large cup-like structure with two ligand-binding sites in its groove. The dimeric complex acts as a novel molecular chaperone that interacts with key molecules involved in cell differentiation, proliferation, transformation, and apoptosis.[1–4] It regulates the function of target proteins by restricting their subcellular location, bridging them to modulate catalytic activity, and protecting them from dephosphorylation or proteolysis.[1–4,8–10] In general, the 14-3-3 protein binds to phosphoserine-containing motifs of the ligands such as RSXpSXP and RXY/FXpSXP in a sequence-specific manner.[1,3,10] More than 100 proteins have been identified as being 14-3-3 binding partners, including a range of intracellular signaling regulators such as Raf, BAD, protein kinase C (PKC), phophatidylinositol 3-kinase (PI3K), and cdc25 phosphatase.[1,4,8–10] Binding of the 14-3-3 protein to Raf is indispensable for Raf kinase activity in the Ras/MAPK signaling pathway, whereas 14-3-3 binding to the mitochondrial Bcl-2 family member BAD, when phosphorylated by a serine/threonine kinase Akt, inhibits apoptosis.[1–4] In addition to the phosphorylation-dependent interaction, the 14-3-3 protein can interact with a set of target proteins in a phosphorylation-independent manner.[10–12] The ε isoform binds to p190RhoGEF via a phosphoserine-independent interaction.[11]

Previous studies indicated that the 14-3-3 protein has isoform-specific and nonredundant functions.[1–4] Synaptic transmission and associative learning are impaired in

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During submission of the present manuscript, an immunohistochemical study (Kawamoto Y, Akiguchi I, Kovacs GG, Hicket H, Budka H: Increased 14-3-3 immunoreactivity in glial elements in patients with multiple sclerosis. Acta Neuropathol 2004, 107:137–143) has been published. This study showed that the 14-3-3 protein is expressed strongly in both astrocytes and oligodendrocytes in MS brains using an anti-14-3-3 protein antibody broadly reactive against all isoforms (H-8, sc-1657, Santa Cruz Biotechnology).

Supplemental information can be found on *http://www.amjpathol.org*.

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Drosophila mutants lacking the ζ protein.[13] The 14-3-3 isoforms have distinct affinities for their target proteins. A preferential interaction is observed between PKCθ and the human 14-3-3θ isoform in T cells,[14] IGF1-receptor, IRS1, and ε isoform,[15] the apoptosis-inhibitor A20 and the human β and η isoforms,[16] and glucocorticoid receptor and the human η isoform.[17] The human β and ζ isoforms and not γ or ε isoforms interact with phosphorylated tau.[18] Furthermore, different isoforms show distinct patterns of spatial, temporal, and subcellular distribution. The human θ and σ isoforms are predominantly expressed in T cells and epithelial cells, respectively.[14,19] The rat ε and γ isoforms are enriched in the synaptosomal membranes,[20] and the γ isoform is the main 14-3-3 protein located in the Golgi apparatus in mammalian cells.[3] In the developing rat brain, defined populations of neurons express β, γ, ζ, and θ isoforms at specific stages of development.[6,7] In the adult mouse brain, β, γ, η, and ζ isoforms are widely distributed with the localization primarily in neurons, although some glial cells express ε, θ, and ζ isoforms.[21]

Recently, several lines of evidence have indicated that the 14-3-3 protein is involved in neurodegenerative processes. The 14-3-3 protein detected in the cerebrospinal fluid of Creutzfeldt-Jacob disease has been used as a biochemical marker for the premortem diagnosis of Creutzfeldt-Jacob disease in the context of differential diagnosis of progressive dementia.[22-24] In addition, intense immunoreactivity against the ζ isoform was identified in amyloid plaques in the Creutzfeldt-Jacob disease brain.[25] However, several studies including our own showed that the 14-3-3 protein is occasionally detectable in the cerebrospinal fluid of infectious meningoencephalitis, metabolic encephalopathy, cerebrovascular diseases, and multiple sclerosis (MS) presenting with severe myelitis, suggesting that it is not a marker specific for prion diseases but for extensive destruction of brain tissues causing the leakage of 14-3-3 protein into the cerebrospinal fluid.[4,22,26,27] In the Alzheimer's disease brain, neurofibrillary tangles express immunoreactivity against the 14-3-3 protein.[28] The 14-3-3ζ homodimer interacts with tau and glycogen synthase kinase-3β (GSK3β), and stimulates GSK3β-mediated tau phosphorylation.[29] In the Parkinson's disease brain, Lewy bodies possess γ, ε, ζ, and θ isoforms that interact with α-synuclein.[30,31] Dopamine-dependent neurotoxicity is mediated by a soluble complex composed of the 14-3-3 protein and α-synuclein, whose levels are markedly elevated in the substantia nigra of the Parkinson's disease brain.[32] The neurotoxicity of ataxin-1, the causative protein of spinocerebellar ataxia type 1, is enhanced by ε and ζ isoforms that bind to and stabilize ataxin-1 phosphorylated by Akt, thereby slowing its degradation.[33] Finally, expression of the θ isoform is enhanced in the spinal cord of amyotrophic lateral sclerosis.[34] However, it remains unknown whether the 14-3-3 protein plays an active role in the pathological process of MS.

In the present study, we investigated the differential expression of seven 14-3-3 isoforms in chronic active demyelinating lesions of MS. We found that reactive astrocytes intensely express β, ε, ζ, η, and σ isoforms, among which the ε isoform provides a specific marker to identify reactive astrocytes in the MS brain. Furthermore, the 14-3-3 protein interacts with vimentin and glial fibrillary acidic protein (GFAP) in cultured human astrocytes. These observations suggest that the 14-3-3 protein plays an organizing role in the intermediate filament (IF) network in reactive astrocytes at the site of demyelinating lesions in MS.

Materials and Methods

MS and Non-MS Brain Tissues

Ten-μ-thick tissue sections were prepared from the brain, spinal cord, and optic nerve derived from four autopsy cases of MS numbered 791, 744, 609, and 544. The clinical and neuroradiological profiles of these patients are shown in a supplementary table on The American

Table 1. The 14-3-3 Isoform-Specific or Broadly Reactive Antibodies Utilized for Immunocytochemistry and Western Blot Analysis

14-3-3 isoforms	Suppliers	Code	Antigen peptide	Origin	Specificity	Concentration used for immunohistochemistry (μg/ml)	Concentration used for Western blotting (μg/ml)
Pan	SC	sc-629	N-terminal	Rabbit	Reactive to all isoforms	0.4	0.04
Pan	SC	sc-1657	N-terminal	Mouse	Reactive to all isoforms	0.4	0.04
β	SC	sc-628	C-terminal	Rabbit	Reactive predominantly to β isoform, but crossreactive to other isoforms to a lesser extent	0.4	0.04
β	IBL	18641	N-terminal	Rabbit	Not crossreactive to other isoforms	2	1
γ	IBL	18647	C-terminal	Rabbit	Not crossreactive to other isoforms	5	0.2
ε	IBL	18643	C-terminal	Rabbit	Not crossreactive to other isoforms	2	1
ζ	IBL	18644	N-terminal	Rabbit	Not crossreactive to other isoforms	2	0.5
η	IBL	18645	N-terminal	Rabbit	Not crossreactive to other isoforms	5	1
θ (τ)	SC	sc-732	C-terminal	Rabbit	Not crossreactive to other isoforms	0.4	0.04
θ (τ)	IBL	10017	Recombinant, whole	Mouse	Minimally crossreactive to σ isoform	1	1
σ	IBL	18642	C-terminal	Rabbit	Not crossreactive to other isoforms	1	1

Abbreviations: SC, Santa Cruz Biotechnology; IBL, Immunobiological Laboratory. The specificity of the antibodies (IBL) is also shown on Supplementary Figure 1 at *http://www.amjpathol.org*.