Background

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) white matter mediated by an autoimmune process, whose development is triggered by a complex interplay of both genetic and environmental factors [1]. Administration of interferon-gamma (IFNγ) induced acute relapses, along with activation of the systemic immune response [2], suggesting that T-lymphocytes producing proinflammatory T helper type 1 (Th1) cytokines play a pivotal role in the immunopathogenesis of MS. In contrast, interferon-beta (IFNβ) significantly reduces the frequency of clinical exacerbations and delays the progression of disability in relapsing-remitting MS (RRMS), accompanied by a reduction in the number of new brain lesions on MRI [3,4]. Furthermore, an early initiation of IFNβ delays the conversion to clinically definite MS in the patients who experienced a first demyelinating event [5]. Although the precise mechanism underlying therapeutic effects of IFNβ on MS remains to be fully elucidated, previous studies proposed several possibilities, including the inhibition of Th1 cell development [6], induction of Th2 immune deviation [7], restoration of function of the disrupted blood-brain barrier [8], and downregulation of IFNγ-induced expression of class II major histocompatibility complex (MHC) molecules [9].

Although clinical benefits of IFNβ in MS are meaningful, approximately one-third of the patients receiving IFNβ therapy suffered from a higher or identical annual relapse rate on treatment [10]. New lesion formation on MRI during the treatment correlates with poor response to IFNβ [11]. Furthermore, a substantial proportion of the patients discontinued IFNβ treatment because of various adverse effects, including skin reactions, flu-like symptoms, leukocytopenia, liver dysfunction, depression and amenorrhea [12]. The molecular mechanisms accounting for IFNβ-related adverse effects remain unknown, although most of these emerge at the early phase after initiation of the treatment, and then diminish with time [12]. At present, no biologically relevant markers capable of predicting either therapeutic or detrimental responses of IFNβ in MS are available [13].

DNA microarray technology is a novel approach that allows us to systematically monitor the expression of a large number of genes. It has given new insights into the complexity of molecular interactions promoting the autoimmune process in MS [14]. IFNβ induces a complex pattern of gene regulation in peripheral blood mononuclear cells (PBMC) of MS [15]. Recently, we studied the gene expression profile of $CD3^+$ T cells isolated from PBMC of 13 Japanese MS patients before and after treatment with IFNβ-1b by analyzing a custom cDNA microarray containing a set of well-annotated, immunologically relevant genes. IFNβ upregulates the expression of 7 IFNβ-responsive genes (IRGs) during the treatment [16]. A following study performed on RRMS patients receiving IFNβ-1a supported our observations [17]. More recently, we found that the vast majority of genes expressed in $CD3^+$ T cells differentially between 72 untreated MS patients and 22 healthy controls are categorized into apoptosis regulators [18]. Regarding the gene expression profile of IFNβ responders in MS, baseline levels of IL-12p35 mRNA are significantly lower in the responders [19]. Downregulation of IL-8 expression in PBMC during IFNβ treatment distinguishes the responders from nonresponders in RRMS [20]. IFNβ responders differ from nonresponders in the kinetics of expression of IRGs at 3 and 6 months after starting the treatment [21]. A three-dimensional model of gene triplets detected by RT-PCR analysis predicts IFNβ response status in RRMS [22]. However, all of previous observations do not clearly illustrate the molecular basis of complex biological effects of IFNβ in MS. Furthermore, no databases of immediate early IRGs in PBMC are currently available.

The present study is designed to identify a comprehensive list of immediate early IRGs in PBMC with potential relevance to IFNβ-related early adverse effects in MS.

Methods

Treatment of peripheral blood lymphocytes with IFNβ

PBMC were isolated from heparinized blood by centrifugation on a Ficoll density gradient. PBMC 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 (culture medium). The cells were incubated in a 5%CO_2/95% air incubator at 37°C for 3 hours to characterize the immediate response or for 24 hours to detect the early response, in the culture medium with or without inclusion of 50 ng/ml recombinant human IFNβ (a specific activity of = 2×10^7 units/mg, PeproTech, London, UK), 50 ng/ml recombinant human IFNγ (a specific activity of = 2×10^7 units/mg, PeproTech), 50 ng/ml recombinant human TNFα (a specific activity of = 2×10^7 units/mg, PeproTech), or 50 ng/ml recombinant human IL-1β (a specific activity of = 1×10^7 units/mg, PeproTech). They were then processed for RNA preparation as described previously [16,18,21]. Written informed consents were obtained from the subjects involved in the present study according to the form approved by the Ethics Committee of National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan. The samples of the subjects #1, #2 and #4 were processed for both microarray and real-time RT-PCR analysis, while those of the subject #3 were studied only by real-time RT-PCR analysis.

Table 1: Primers utilized for real-time RT-PCR analysis

Genes	GenBank accession No.	Sense primers	Antisense primers	PCR product (bp)
ISG15 (G1P2)	NM_005101 (M13755)	5'aagcccctgagcaccgtgttcat3'	5'ttgatcctgctcggatgctggtg3'	102
SCYB10 (CXCL10, IP-10)	NM_001565 (X02530)	5'tcgatgcagtgcttccaaggatgg3'	5'ccttcctacaggagtagtagcagc3'	162
SCYA8 (CCL8, MCP2)	NM_005623 (Y10802)	5'tctgtgctgaccccaaggagagat3'	5'taatgtcacactgcacctgggga3'	164
SCYA2 (CCL2, MCP1)	NM_002982 (S71513)	5'ctagctttccccagacaccctgtt3'	5'ccaggggtagaactgtggttcaag3'	197
SCYB2 (CXCL2, GRO2)	NM_002089	5'cccgcatcgcccatggttaagaaa3'	5'tcttctgttcctgtaaggcagggc3'	131
FOS	NM_005252	5'gagctggtgcattacagagaggag3'	5'ggacttgagtccacacatggatgc3'	140
RGS14	NM_006480	5'tgacagctacccaacagtccagga3'	5'agggattgggggtgagcttgttga3'	222
G3PDH	NM_002046	5'ccatgttcgtcatgggtgtgaacca3'	5'gccagtagaggcagggatgatgttc3'	251

Abbreviations: ISG15, interferon-stimulated gene 15; SCYB10, small inducible cytokine subfamily B, member 10; SCYA8, small inducible cytokine subfamily A, member 8; SCYA2, small inducible cytokine subfamily A, member 2; SCYB2, small inducible cytokine subfamily B, member 2; FOS, cellular oncogene c-fos; RGS14, regulator of G-protein signaling 14; and G3PDH, glyceraldehyde-3-phosphate dehydrogenase

Quantitative real-time RT-PCR analysis

DNase-treated total RNA was processed for cDNA synthesis using oligo(dT)$_{12-18}$ primers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). cDNA was amplified by PCR in LightCycler ST300 (Roche Diagnostics, Tokyo, Japan) using SYBR Green I dye and the primer sets listed in Table 1. To calibrate the concentration of mRNA levels in test cDNA samples, serially-diluted purified PCR products generated by conventional RT-PCR (a 10-fold dilution from 1 pg/ml to 0.0001 pg/ml) were amplified in parallel. The levels of expression of target genes were standardized against those of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene detected in the identical cDNA samples. The assays were performed in triplicate measurements of the same sample and the results were expressed as the average with standard error.

cDNA microarray analysis

The present study utilized a custom microarray containing duplicate spots of 1,258 cDNA immobilized on a poly-L-lysine-coated slide glass (Hitachi Life Science, Kawagoe, Saitama, Japan) [16,18,21]. They were prepared by PCR of well-annotated genes, selected from cytokines, growth factors and their receptors, apoptosis regulators, oncogenes, transcription factors, cell cycle regulators and housekeeping genes. The complete gene list is shown in Additional file 1. Five μg of purified RNA was *in vitro* amplified and antisense RNA (aRNA) was processed for microarray analysis. aRNA derived from IFNβ-treated PBMC was labeled with a fluorescent dye Cy5, while aRNA of untreated PBMC was labeled with Cy3 by reverse transcriptase reaction. The arrays were hybridized at 62°C for 17 hours in the hybridization buffer containing equal amounts of Cy3- or Cy5-labeled cDNA, and they were then scanned at two different photomultiplier tube (PMT) gains by the ScanArray 5000 scanner (GSI Lumonics, Boston, MA). The data were analyzed by using the QuantArray software (GSI Lumonics). The fluorescence intensities (FI) of individual spots were quantified following global normalization between Cy3 and Cy5 signals. The average of FI of duplicate spots was calculated, then the ratio of FI of Cy5 signal versus FI of Cy3 signal exceeding 2.0 was defined as significant upregulation, whereas the ratio smaller than 0.5 was considered as substantial downregulation. The impact of inter-experiment variability was validated by analyzing a scatter plot (see Additional file 2).

The IFN-regulated expression of the genes we identified was verified by computerized search of PubMed database and IFN Stimulated Gene (ISG) database [23].

Results

Microarray analysis identified immediate early IFNβ-responsive genes in PBMC

Among 1,258 genes on the array, IFNβ treatment for 3 hours elevated the expression of 107 genes in PBMC isolated from a 46 year-old healthy man (the subject #1), while it reduced the expression of 22 genes (see Additional files 3 and 4 for the complete list). IFNβ treatment for 24 hours upregulated 87 genes and downregulated 23 genes (see Additional files 5 and 6 for the complete list). Sixty-nine genes were upregulated at both 3 and 24 hours, while only two genes such as FOS and IL1A were downregulated at both. The IRGs upregulated at both time points contained 11 *in vivo* IRGs reported previously by us [16], including IFIT1 (IFI56), ISG15 (G1P2), IFIT4 (IFI60), IFI27, G1P3 (IFI6-16), IRF7, ABCB2 (TAP1), ATF3, IFITM1 (IFI17), SULT1C1, and TNFAIP6, whose expression was elevated in T cells and non-T cells *ex vivo*, isolated from 13 RRMS patients during IFNβ treatment for 3 to 6 months. Top 20 most significant genes, either

Table 2: Top 20 upregulated genes in PBMC following exposue to IFNβ

No.	Cy5/Cy3 ratio	Symbol	GenBank	Gene name
at 3 hours				
1	188.50	IFIT1	X03557	interferon-induced protein with tetratricopeptide repeats 1 (IFI56)
2	149.82	SCYB11	AF030514	chemokine (C-X-C motif) ligand 11 (CXCL11, IP-9, I-TAC)
3	81.81	ISG15	M13755	interferon-stimulated gene ISG15 (G1P2)
4	67.84	IFIT4	AF083470	interferon-induced protein with tetratricopeptide repeats 4 (IFI60)
5	66.38	MX1	M30817	myxovirus resistance protein 1 (MXA)
6	64.43	SCYB10	X02530	chemokine (C-X-C motif) ligand 10 (CXCL10, IP-10)
7	55.01	SCYA8	Y10802	chemokine (C-C motif) ligand 8 (CCL8, MCP2)
8	36.88	SCYB9	X72755	chemokine (C-X-C motif) ligand 9 (CXCL9, MIG)
9	35.14	TNFSF10	U37518	tumor necrosis factor superfamily, member 10 (TRAIL)
10	32.86	MX2	M30818	myxovirus resistance protein 2 (MXB)
11	21.99	IFI27	X67325	interferon alpha-inducible protein 27
12	21.98	G1P3	X02492	interferon alpha-inducible protein (IFI6-16), isoform a
13	19.26	ISG20	U88964	interferon-stimulated gene ISG20
14	19.12	STAT1	M97935	signal transducer and activator of transcription 1, isoform alpha
15	13.71	IRF7	U53830	interferon-regulatory factor 7, isoform alpha
16	13.44	CCNA1	U66838	cyclin A1
17	12.72	IL6	M14584	interleukin 6
18	10.86	IL1RN	X53296	interleukin 1 receptor antagonist, isoform 1
19	10.47	SCYA2	S71513	chemokine (C-C motif) ligand 2 (CCL2, MCP1)
20	9.91	STAT2	M97934	signal transducer and activator of transcription 2
at 24 hours				
1	193.19	SCYA8	Y10802	chemokine (C-C motif) ligand 8 (CCL8, MCP2)
2	124.77	IFIT1	X03557	interferon-induced protein with tetratricopeptide repeats 1 (IFI56)
3	107.21	IFI27	X67325	interferon alpha-inducible protein 27
4	70.51	ISG15	M13755	interferon-stimulated gene ISG15 (G1P2)
5	53.22	SCYB11	AF030514	chemokine (C-X-C motif) ligand 11 (CXCL11, IP-9, I-TAC)
6	38.07	MX1	M30817	myxovirus resistance protein 1 (MXA)
7	37.95	IFIT4	AF083470	interferon-induced protein with tetratricopeptide repeats 4 (IFI60)
8	31.56	SCYB10	X02530	chemokine (C-X-C motif) ligand 10 (CXCL10, IP-10)
9	23.65	G1P3	X02492	interferon alpha-inducible protein (IFI6-16), isoform a
10	23.10	TNFSF10	U37518	tumor necrosis factor superfamily, member 10 (TRAIL)
11	21.31	MX2	M30818	myxovirus resistance protein 2 (MXB)
12	20.68	PDGFRL	D37965	platelet-derived growth factor receptor-like protein
13	15.95	CD80	M27533	CD80 antigen (B7-1)
14	13.99	IRF7	U53830	interferon-regulatory factor 7, isoform alpha
15	13.93	IL1RN	X53296	interleukin 1 receptor antagonist, isoform 1
16	13.31	ISG20	U88964	interferon-stimulated gene ISG20
17	11.81	IFITM1	J04164	interferon induced transmembrane protein 1 (IFI17)
18	8.67	SCYA2	S71513	chemokine (C-C motif) ligand 2 (CCL2, MCP1)
19	8.54	IL6	M14584	interleukin 6
20	8.51	IL8RB	L19593	interleukin 8 receptor beta (CXCR2)

Top 20 upregulated genes in PBMC of the subject #1 (a 46 year-old healthy man) by exposure to 50 ng/ml recombinant human IFNβ for 3 or 24 hours are listed with Cy5/Cy3 signal intensity ratio, gene symbol, GenBank accession number, and gene name. In vivo IRGs in T cells and non-T cells of RRMS patients reported previously (Ref. 16) are underlined.

upregulated or downregulated, are listed in Table 2 and Table 3, respectively. All of top 20 upregulated genes were found as known IRGs identified in various cell types by searching through PubMed and ISG databases.

The upregulated IRGs in the complete lists (see Additional files 3 and 5) were classified into several functional categories following; (i) conventional IFN-response markers (n = 12), (ii) components of classical and Toll-like receptor (TLR)-dependent IFN-signaling pathways (n = 12), (iii) chemokines and their receptors (n = 11), (iv) cytokines, growth factors and their receptors (n = 17), (v) apoptosis, DNA damage, and cell cycle regulators (n = 29), (vi) heat shock proteins (n = 9), and (vii) costimulatory and adhesion molecules (n = 7) (Table 4). The chemokine and chemokine receptor group included both CXC and CC chemokines and their receptors, such as SCYB11 (CXCL11, I-TAC), SCYB10 (CXCL10, IP-10), SCYA8 (CCL8, MCP2), SCYB9 (CXCL9, MIG), SCYA2 (CCL2, MCP1), CCR5, SCYA4 (CCL4, MIP1B), IL8RB (CXCR2),

Table 3: Top 20 downregulated genes in PBMC following exposue to IFNβ

No.	Cy5/Cy3 ratio	Symbol	GenBank	Gene name
at 3 hours				
1	0.29	RGS14	NM_006480	regulator of G-protein signaling 14
2	0.30	FOS	NM_005252	cellular oncogene c-fos
3	0.31	SCYB2	NM_002089	chemokine (C-X-C motif) ligand 2 (GRO2, MIP2A)
4	0.41	PPARG2	U63415	peroxisome proliferative activated receptor gamma, isoform 2
5	0.41	TNFSF11	NM_003701	tumor necrosis factor ligand superfamily, member 11 (RANKL), isoform 1
6	0.42	ABCC4	NM_005845	ATP-binding cassette, subfamily C, member 4 (MRP4)
7	0.42	MERTK	NM_006343	c-mer proto-oncogene tyrosine kinase
8	0.42	TGFBR3	NM_003243	transforming growth factor beta receptor III (betaglycan)
9	0.44	ATP2B1	J04027	ATPase, Ca++ transporting, plasma membrane 1, isoform 1b
10	0.45	SCYB1	NM_001511	chemokine (C-X-C motif) ligand 1 (GRO1, MGSA)
11	0.45	IL8	NM_000584	interleukin 8 (SCYB8, CXCL8)
12	0.45	TRAF5	NM_004619	TNF receptor-associated factor 5, variant 1
13	0.47	CD3G	NM_000073	CD3G antigen, gamma polypeptide
14	0.47	IL1A	NM_000575	interleukin 1 alpha
15	0.47	TCFL5	NM_006602	transcription factor-like 5
16	0.48	LAT	AF036905	linker for activation of T cells, isoform b
17	0.48	HNMT	U08092	histamine N-methyltransferase, isoform 1
18	0.48	SLC3A1	NM_000341	solute carrier family 3, member 1
19	0.49	MGST1	NM_145764	microsomal glutathione S-transferase 1, variant 1d
20	0.49	TNFSF8	NM_001244	tumor necrosis factor ligand superfamily, member 8 (CD30L)
at 24 hours				
1	0.18	ABCC3	AF083552	ATP-binding cassette, subfamily C, member 3, isoform MRP3
2	0.20	FOS	NM_005252	cellular oncogene c-fos
3	0.30	ALDH2	NM_000690	aldehyde dehydrogenase 2 family
4	0.30	TPST1	NM_003596	tyrosylprotein sulfotransferase 1
5	0.31	CD9	NM_001769	CD9 antigen
6	0.34	NRG1	NM_013962	neuregulin 1, isoform GGF2
7	0.35	TLR5	NM_003268	toll-like receptor 5
8	0.36	PTGS1	NM_000962	prostaglandin-endoperoxide synthase 1 (COX1), isoform 1
9	0.37	MGST2	NM_002413	microsomal glutathione S-transferase 2
10	0.38	ITGAM	NM_000632	integrin, alpha M (CD11B, MAC-1, CR3A)
11	0.38	SCYA24	NM_002991	chemokine (C-C motif) ligand 24 (eotaxin 2)
12	0.38	CYP27A1	NM_000784	cytochrome P450, family 27, subfamily A, polypeptide 1 (CTX)
13	0.39	ALDH1A1	NM_000689	aldehyde dehydrogenase 1 family, member A1
14	0.39	MYCL1	NM_005376	v-myc myelocytomatosis viral oncogene homolog 1 (LMYC)
15	0.41	ATP6AP2	NM_005765	ATPase, H+ transporting, lysosomal accessory protein 2 (ATP6M8-9)
16	0.44	PRKCG	NM_002739	protein kinase C gamma
17	0.45	ATP2B1	J04027	ATPase, Ca++ transporting, plasma membrane 1, isoform 1b
18	0.46	AP1S2	NM_003916	adaptor-related protein complex 1, sigma 2 subunit
19	0.47	NR6A1	NM_001489	nuclear receptor subfamily 6, group A, member 1 (RTR), isoform 2
20	0.47	GCG	NM_173916	glucagon

Top 20 downregulated genes in PBMC of the subject #1 (a 46 year-old healthy man) by exposure to 50 ng/ml recombinant human IFNβ for 3 or 24 hours are listed with Cy5/Cy3 signal intensity ratio, gene symbol, GenBank accession number, and gene name.

SCYA3 (CCL3, MIP1A), SCYA19 (CCL19, MIP3B) and SCYA13 (CCL13, MCP4). It is worthy to note that both CXCR3 ligand chemokines (SCYB11, SCYB10 and SCYB9) and CCR2 ligand chemokines (SCYA10 and SCYA2) were clustered in top 20 genes greatly elevated at 3 and 24 hours of IFNβ treatment (Table 2). With respect to top 20 downregulated genes, four genes such as SCYB2 (CXCL2, GRO2), SCYB1 (CXCL1, GRO1), IL8 (SCYB8, CXCL8), and SCYA24 (eotaxin-2) were categorized into the chemokine group (Table 3). Among them, SCYB2, SCYB1 and IL8, whose expression was reduced immediately at 3 hours of IFNβ treatment, belong to CXCR2 ligand chemokines. Although the analysis in the present study was a single microarray for each sample design, the results from two additional subjects, including a 28 year-old healthy man (the subject #2) and a 27 year-old woman with RRMS who was a dropout of IFNβ treatment due to induction of frequent severe relapses (the subject #4), verified the observations of immediate early induction of CXCR3 ligand and CCR2 ligand chemokine genes in PBMC by exposure to IFNβ, supporting the reproducibility of these results (see Additional file 7).

Table 4: Functional classification of IFNβ-upregulated genes in PBMC

Categories	Number of genes	Gene symbols (alternative symbols or names)
1 **Conventional IFN-response markers**	12	IFIT1 (IFI56), ISG15 (G1P2), IFIT4 (IFI60), MX1 (MXA), MX2 (MXB), IFI27, G1P3 (IFI6-16), ISG20, IFI16, IFITM1 (IFI17), IFITM3 (1-8U), ABCB2 (TAP1)
2 **Components of IFN-signaling pathways**	12	STAT1, IRF7, STAT2, JAK2, IRF2, ISGF3G (IRF9), MYD88, IRF8, STAT3, JAK3, IRF1, TLR3
3 **Chemokines and receptors**	11	SCYB11 (CXCL11, I-TAC), SCYB10 (CXCL10, IP-10), SCYA8 (CCL8, MCP2), SCYB9 (CXCL9, MIG), SCYA2 (CCL2, MCP1), CCR5, SCYA4 (CCL4, MIP1B), IL8RB (CXCR2), SCYA3 (CCL3, MIP1A), SCYA19 (CCL19, MIP3B), SCYA13 (CCL13, MCP4)
4 **Cytokines, growth factors, and receptors**	17	IL6, ILRN (IL-1 receptor antagonist), IL1R2, IL15RA, IL15, SPP1 (osteopontin), CSF1, IL12RB2, TNF (TNFA), IL2RB, IFNG, NTRK1 (TRKA), PDGFRL, TNFAIP6, KITLG (SCF), IL10, IL3RA
5 **Apotosis, DNA damage, and cell cycle regulators**	29	TNFSF10 (TRAIL), CASP10, BAG1, TNFRSF6 (FAS), CASP4, TRADD, GZMA, CASP7, RIPK2, MAD, RIPK1, CFLAR (FLIP), RELA, STK3, CASP1, TNFSF6 (FASL), PARP4, TANK (I-TRAF), POLE2, LMNB1, E2F2, CCNA1 (cyclin A1), CDKN1A (p21), PPP1R15A (GADD34), CASP3, CDKN1C (p57), CDK5R2 (p39), TERF1, NBS1 (nibrin)
6 **Heat shock proteins**	9	HSPA6 (HSP70B'), HSJ2 (HSPF4), HSPA1A (HSP70-1), HSPA1B (HSP70-2), HSPCA (HSP90A), HSPA5 (GRP78), HSPA1L (HSP70-HOM), HSPA8 (HSC70), HSPB1 (HSP27)
7 **Costimulatory and adhesion molecules**	7	CD80 (B7-1), SELL (selectin L), TNFRSF5 (CD40), CD163, CD86 (B7-2), HLA-DRA, FCER1G

The upregulated IRGs in PBMC listed in Additional files 3 and 5 were classified into seven functional categories.

Real-time RT-PCR analysis validated IFNβ-regulated expression of IRGs identified by microarray analysis

Although the microarray we utilized contains total 64 spots of the G3PDH gene (see Additional file 1), G3PDH was neither identified as a significantly upregulated nor a downregulated gene in the microarray analysis, suggesting that G3PDH represents a reliable housekeeping gene in gene expression analysis of PBMC following treatment with IFNβ. Therefore, quantitative real-time RT-PCR analysis was performed by evaluating the levels of expression of target genes standardized against those of G3PDH detected in the identical cDNA samples. It verified the key observations of microarray analysis, such as marked upregulation of ISG15, the prototype of IRGs (Figure 1a–c), and great elevation of SCYB10, SCYA8 and SCYA2 (Figures 2, 3, 4a–c) in PBMC at both 3 and 24 hours of IFNβ treatment. Furthermore, the quantitative analysis confirmed substantial downregulation of FOS at both time points (Figure 5a–c), and RGS14 and SCYB2 predominantly at 3 hours (Figures 6, 7a–c). Exposure of PBMC to IFNγ greatly elevated the expression of SCYB10 and SCYA2, and to a lessor extent, ISG15 and SCYA8 at both time points (Figures 1, 2, 3, 4d), suggesting a functional overlap in induction of CXCR3 ligand and CCR2 ligand chemokines between type I and type II IFN signaling pathways. In contrast, TNFα and IL-1β the prototype of proinflammatory cytokines, did not at all elevate the levels of expression of ISG15, SCYB10 or SCYA8 (Figures 1, 2, 3e, f), while IL-1β significantly (p = 0.041 at 3 hours and p = 0.004 at 24 hours by two-sided paired t-test) but TNFα only marginally (p = 0.2102 at 3 hours and p = 0.0825 at 24 hours by two-sided paired t-test) upregulated SCYA2 expression (Figure 4e, f). Treatment with IFNγ, TNFα or IL-1β reduced the levels of FOS and RGS14 substantially at 24 hours (Figures 5, 6d–f). IFNγ reduced the expression of SCYB2, whereas TNFα and IL-1β elevated its levels at both time points, suggesting differential regulation of SCYB2 gene expression in PBMC by exposure to distinct cytokines (Figure 7d–f). The IFNβ-regulated gene expression pattern was similar among PBMC derived from three distinct healthy subjects #1, #2 and #3, supporting the reproducibility of these observations (Figures 1, 2, 3, 4, 5, 6, 7a–c).

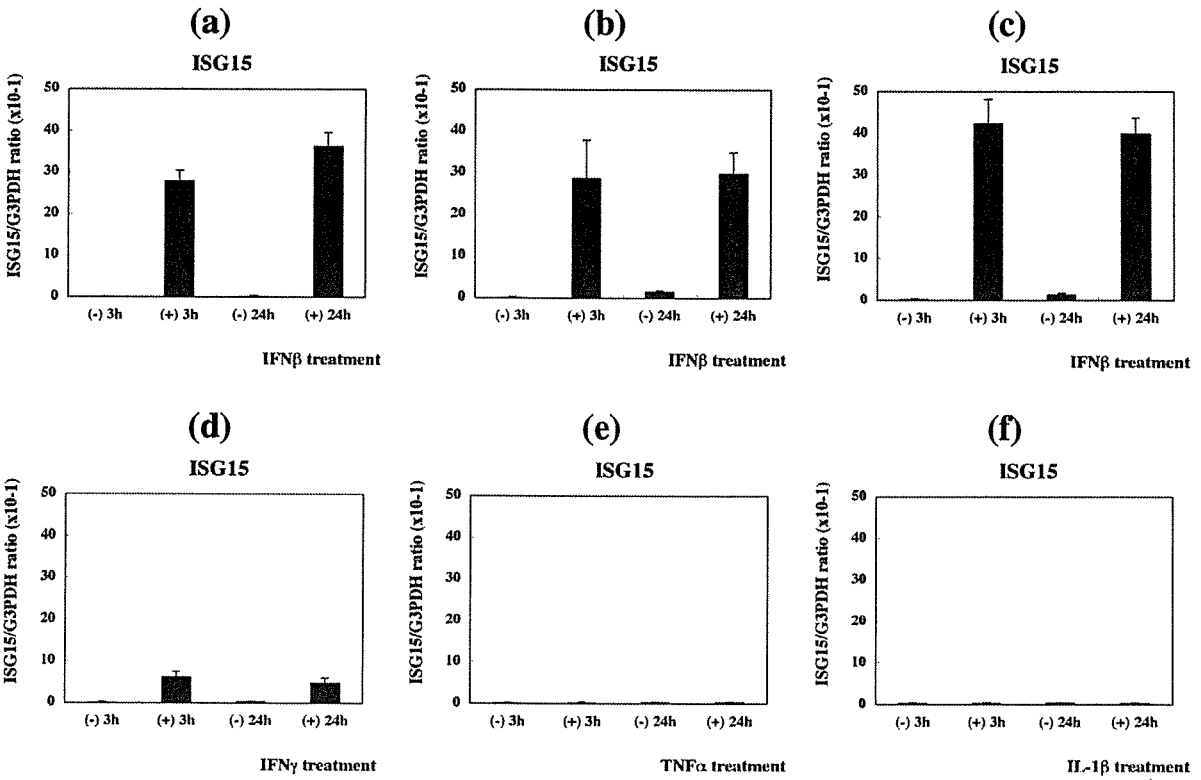

Figure 1

Real-time RT-PCR analysis of ISG15 expression in PBMC. PBMC derived from three distinct healthy subjects numbered #1 (a 46 year-old man), #2 (a 28 year-old man), and #3 (a 42 year-old woman) were incubated for 3 hours or 24 hours in the culture medium with (+) or without (-) inclusion of recombinant human IFNβ, IFNγ, TNFα or IL-1β at a concentration of 50 ng/ml each. cDNA was processed for real-time PCR analysis using specific primers listed in Table 1. The levels of expression of ISG15 are standardized against those of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene detected in identical cDNA samples. The assays were performed in triplicate measurements of the same sample, and the results were expressed as the average with standard error. The panels represent the expression of ISG15 in (a) #1, IFNβ; (b) #2, IFNβ; (c) #3, IFNβ; (d) #1, IFNγ; (e) #1, TNFα; and (f) #1, IL-1β.

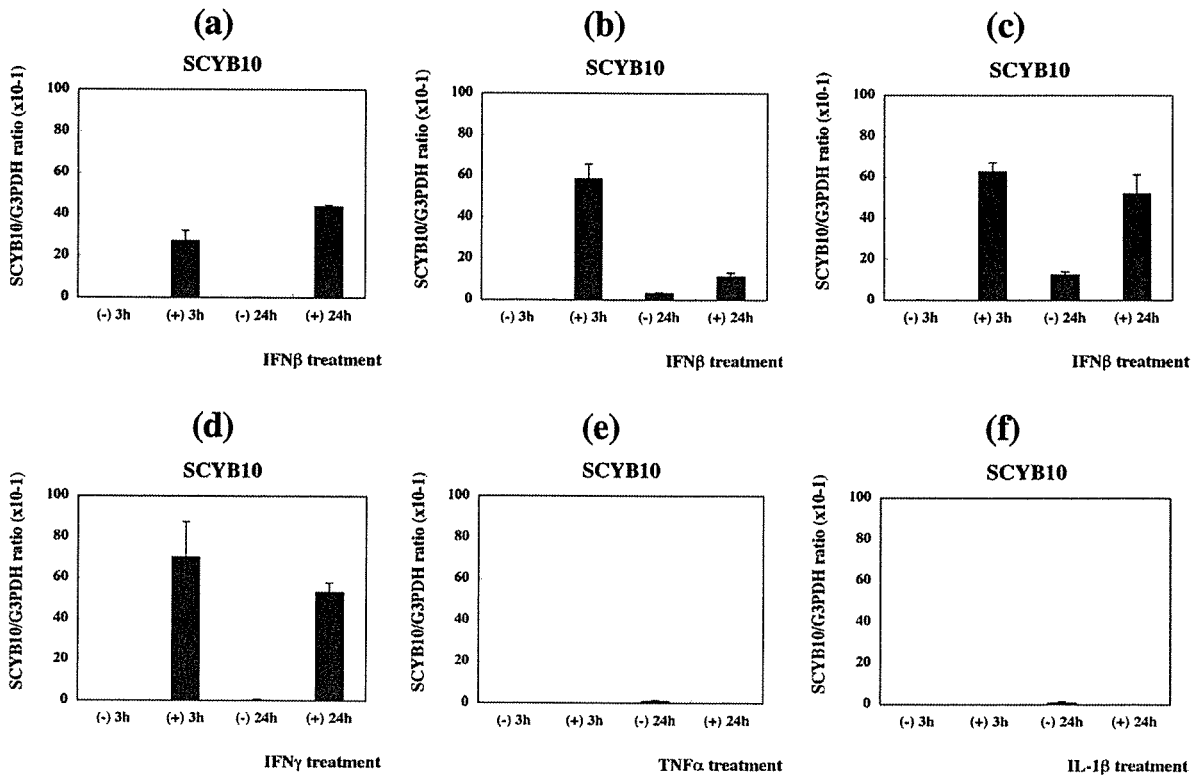

Figure 2

Real-time RT-PCR analysis of SCYB10 expression in PBMC. See the footnote of Figure 1. The panels represent the expression of SCYB10 in (a) #1, IFNβ; (b) #2, IFNβ; (c) #3, IFNβ; (d) #1, IFNγ; (e) #1, TNFα; and (f) #1, IL-1β.

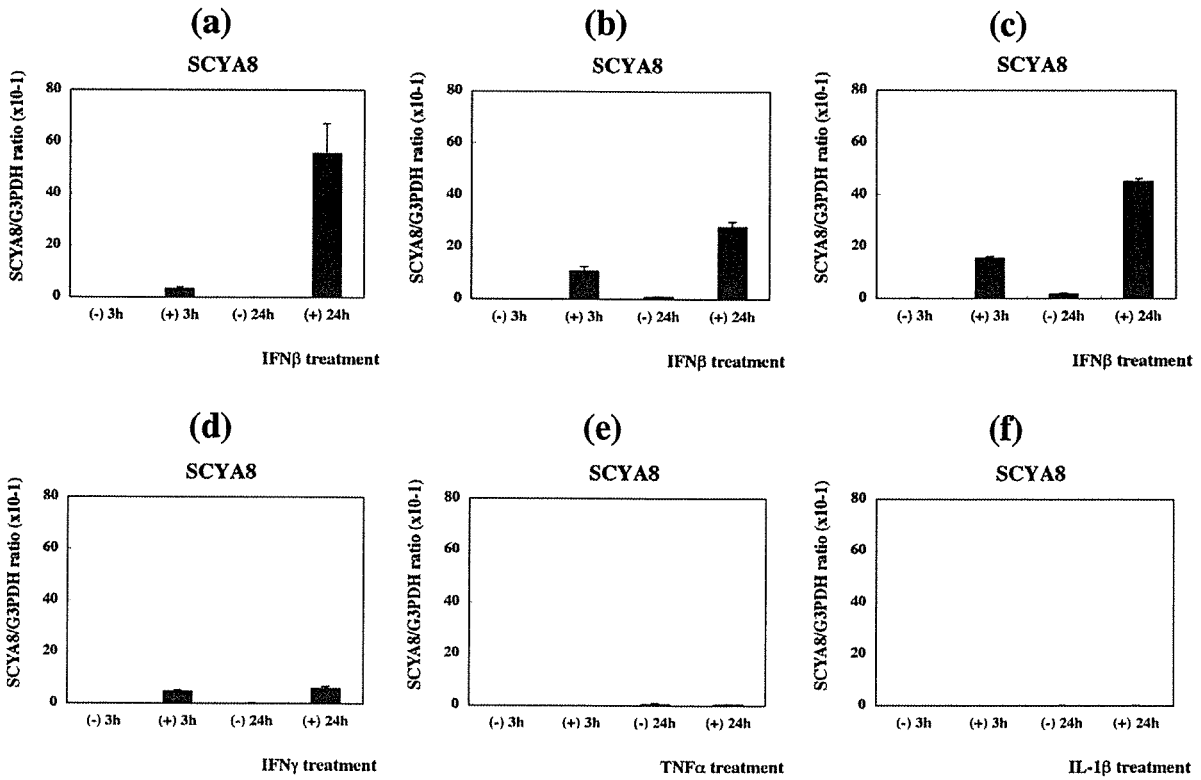

Figure 3

Real-time RT-PCR analysis of SCYA8 expression in PBMC. See the footnote of Figure 1. The panels represent the expression of SCYA8 in (a) #1, IFNβ; (b) #2, IFNβ; (c) #3, IFNβ; (d) #1, IFNγ; (e) #1, TNFα; and (f) #1, IL-1β.

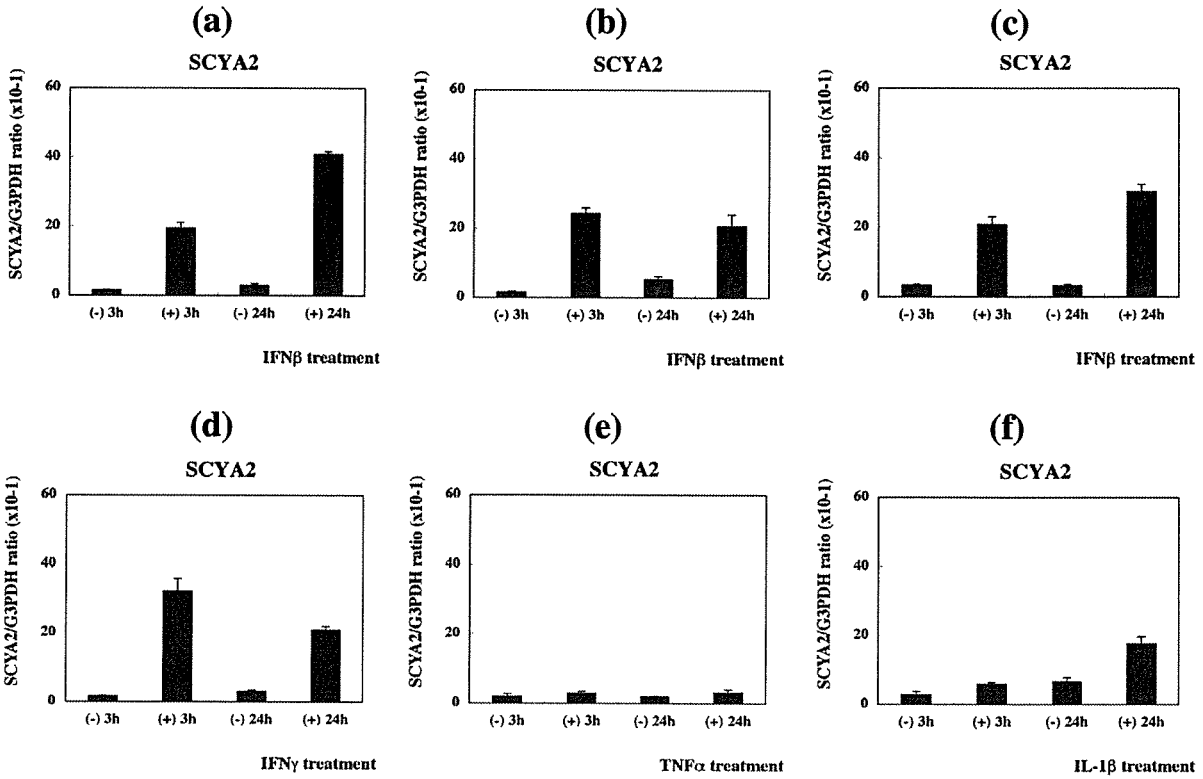

Figure 4

Real-time RT-PCR analysis of SCYA2 expression in PBMC. See the footnote of Figure 1. The panels represent the expression of SCYA2 in (a) #1, IFNβ; (b) #2, IFNβ; (c) #3, IFNβ; (d) #1, IFNγ; (e) #1, TNFα; and (f) #1, IL-1β.

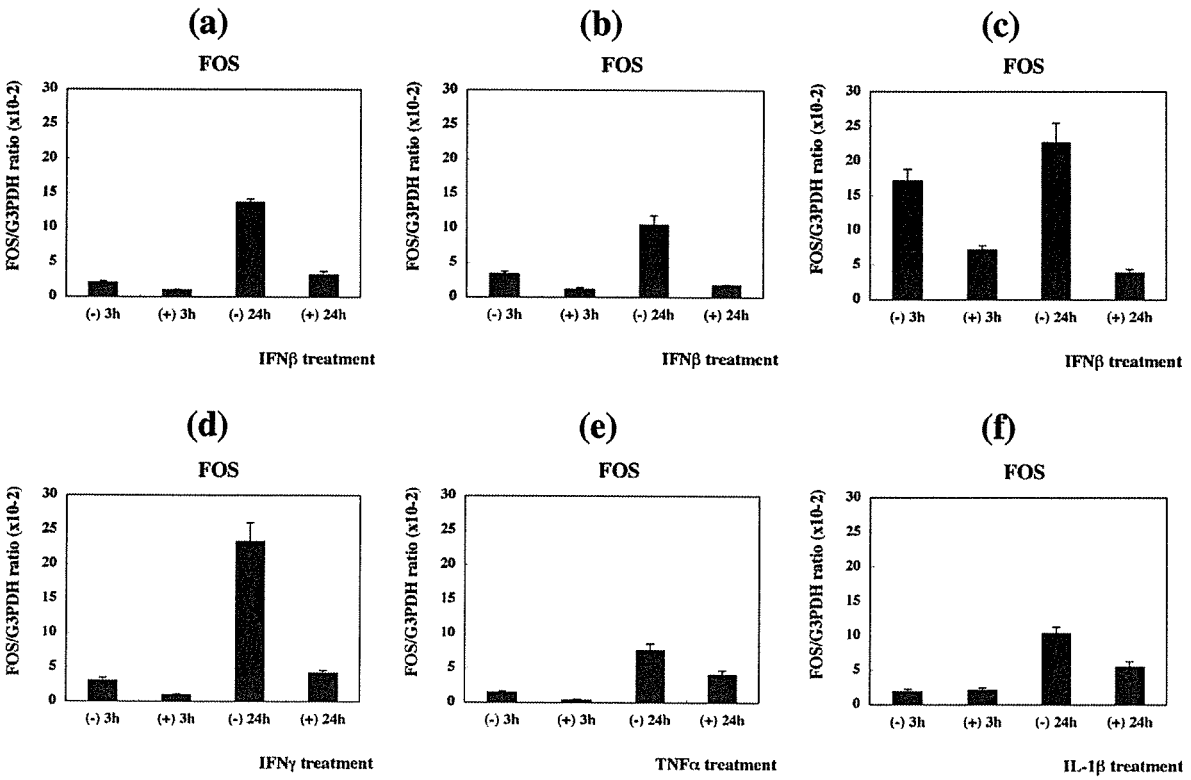

Figure 5

Real-time RT-PCR analysis of FOS expression in PBMC. See the footnote of Figure 1. The panels represent the expression of FOS in (a) #1, IFNβ; (b) #2, IFNβ; (c) #3, IFNβ; (d) #1, IFNγ; (e) #1, TNFα; and (f) #1, IL-1β.

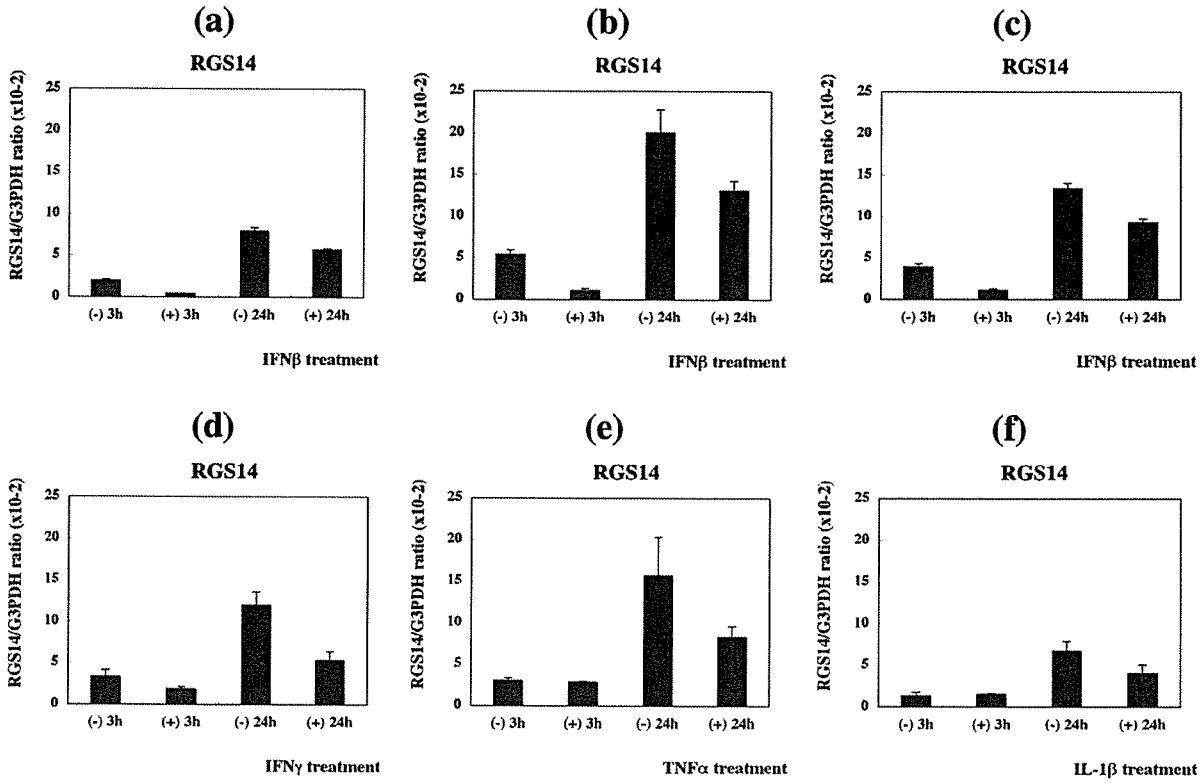

Figure 6

Real-time RT-PCR analysis of RGS14 expression in PBMC. See the footnote of Figure 1. The panels represent the expression of RGS14 in (a) #1, IFNβ; (b) #2, IFNβ; (c) #3, IFNβ; (d) #1, IFNγ; (e) #1, TNFα; and (f) #1, IL-1β.

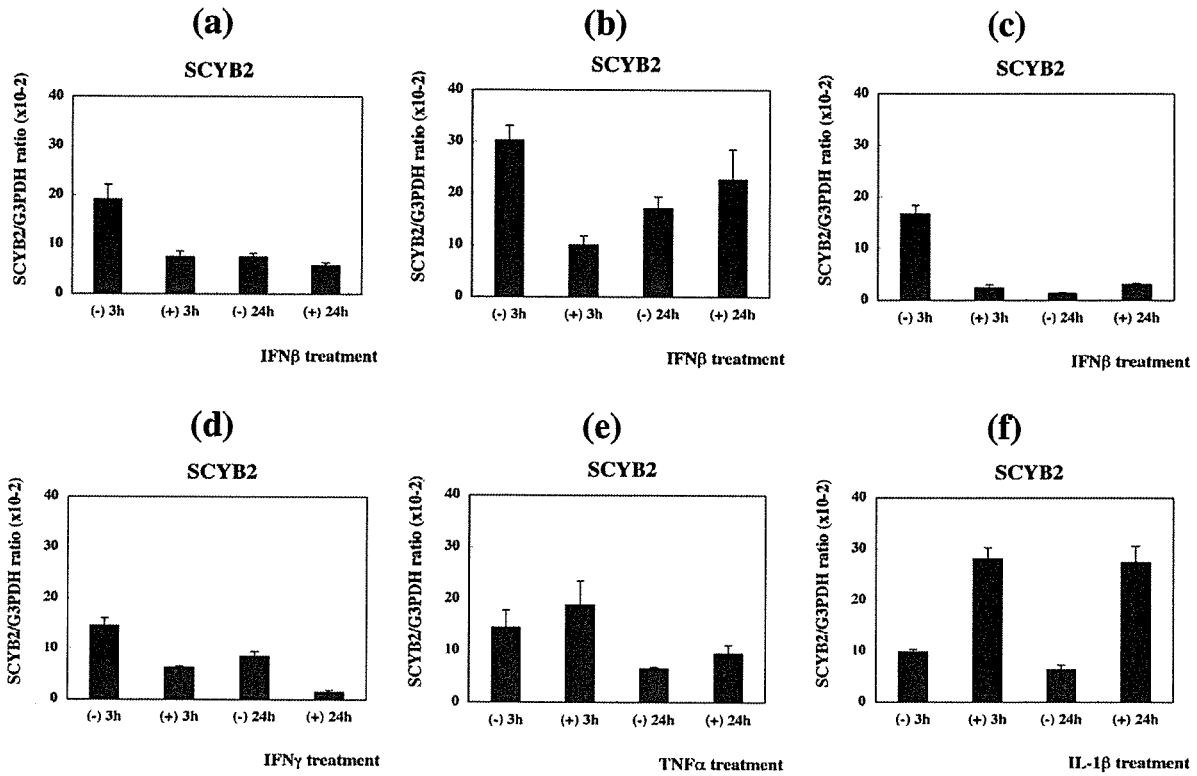

Figure 7
Real-time RT-PCR analysis of SCYB2 expression in PBMC. See the footnote of Figure 1. The panels represent the expression of SCYB2 in (a) #1, IFNβ; (b) #2, IFNβ; (c) #3, IFNβ; (d) #1, IFNγ; (e) #1, TNFα; and (f) #1, IL-1β.

Discussion

IFNs are a family of cytokines that mediates antiviral, antiproliferative and immunoregulatory activities. Type I IFNs, IFNα and β, are produced principally by virus-infected host cells, whereas type II IFN, IFNγ, is produced by activated T cells and natural killer (NK) cells. Type I IFNs activate JAK protein tyrosine kinases associated with the cell surface receptors for IFNs, leading to formation of the complex of signal transducer and activator of transcription (STAT) molecules with the IFN regulatory factor (IRF) family of transcription factors. The STAT/IRF complex translocates into the nucleus, and binds to the DNA sequences termed the IFN-stimulated response element (ISRE) or the IRF-recognition element (IRE). This binding subsequently activates transcription of a wide variety of IFN-responsive genes (IRGs) as well as the genes of type I and type II IFNs, leading to the biological responses triggered by the IFNs [24]. Both type I and type II IFNs enhance the expression of class I and class II MHC molecules [25]. Among nine distinct IRFs, IRF7 and IRF3 play a central role in induction of type I IFN genes via the virus-activated MYD88-independent pathway or Toll-like receptor (TLR)7, 8 or 9-activated MYD88-dependent pathway [26], while IRF1 plays more active roles in induction of IFNγ-target genes essential for Th1-type immune response [25].

The present study by analyzing DNA microarray characterized a comprehensive list of immediate early IRGs in PBMC *in vitro*. Following a 3 to 24 hour-exposure to IFNβ, upregulated genes greatly outnumbered downregulated genes. All top 20 upregulated genes represent known IRGs previously identified in various cell types. The upregulated IRGs of PBMC were classified into several functional categories. The list included not only conventional IFN-response markers and components of IFN-signaling pathways, but also contained many proinflammatory chemokines and cytokines. This is surprising because IFNβ acts principally as an anti-Th1, anti-inflammatory cytokine [6,7]. By analyzing global gene expression profile, the present study for the first time showed that IFNβ induced a burst of gene expression of CXCR3 ligand chemokines

(SCYB11, SCYB10 and SCYB9) and CCR2 ligand chemokines (SCYA8 and SCYA2), which was verified by quantitative real-time RT-PCR analysis. The chemokine genes actually have ISRE or IRE in the promoter regions, indicating direct targets of IFNβ[27,28].

CXCR3 is expressed predominantly on activated Th1 T cells, while CCR2 is expressed chiefly on monocytes [29]. The number of CXCR3+T cells is increased in the blood of RRMS, and they accumulate in perivascular infiltrates in active MS lesions [30,31], while SCYB10 (IP-10) and SCYB9 (MIG) are detected in the cerebrospinal fluid (CSF) of RRMS at acute relapse and expressed in reactive astrocytes in active MS lesions [31,32]. SCYA2 (MCP1) and SCYA8 (MCP2) immunoreactivities are also identified in reactive astrocytes in active demyelinating lesions of MS [33,34]. These observations suggest that CXCR3, CCR2, and their ligand chemokines positively regulate active inflammation in MS. Although the precise cell types expressing CXCR3 ligand and CCR2 ligand chemokines in PBMC in response to IFNβ remain to be characterized, the chemokine burst plays a central role in rapid activation and systemic recruitment of Th1 T cells and monocytes immediately after initiation of IFNβ treatment. A recent study showed that IFNβ promotes trafficking of mouse leukocytes by regulating a specific set of chemokines [35]. However, concurrent upregulation of a set of CXCR3 and CCR2 ligand chemokines has not previously been reported in MS patients on a long-term IFNβ treatment [16,17,20-22], suggesting that this phenomenon is an immediate early but transient event *in vivo*. IFNβ immediately reduced the expression of RGS14 (the most significantly downregulated gene at 3 hours; see Table 3), a member of the regulator of G protein signaling (RGS) gene family that acts as a negative regulator of G protein-coupled receptor (GPCR) signaling. Since all chemokine receptors are GPCR, IFNβ-induced downregulation of RGS14 might facilitate chemokine responsiveness in the cells expressing RGS14 [36,37]. Much less is known about the mechanism for regulation of IFNβ-repressed genes [38]. We identified IL-8 as one of IFNβ-repressed genes in PBMC (Table 3). IFNβ inhibits the transcription of IL-8 gene, possibly by binding of NF-κB repressing factor (NRF) to a negative regulatory element of the IL-8 promoter [39]. Serum IL-8 levels and IL-8 secretion from PBMC are elevated in untreated MS, and then reduced following IFNβ therapy [40]. Downregulation of IL-8 expression in PBMC during IFNβ treatment provides a predictive indicator for the responders in RRMS [20].

IFNβ also promptly upregulated a variety of proinflammatory cytokines, such as IL-6, IL-15, osteopontin, TNFα, and IFNγ in PBMC (Table 4). IFNβ promotes production of TNFα and IFNγ in unstimulated PBMC but decreases their levels in preactivated PBMC [41-43]. IFNβ increases the number of IFNγ-secreting cells *in vivo* at the early period of the treatment [44]. Most importantly, proinflammatory cytokines and chemokines induced by IFNβ have relevance to treatment-related early adverse effects. There exists a close relationship between flu-like symptoms and increased levels of IL-6 [45]. A single injection of IFNβ induces a transient burst of SCYB10 (IP-10) in the plasma of RRMS patients, which correlates with an incidence of flu-like symptoms [46]. IFNβ enhances the expression of CD80, SCYB10 (IP-10) and SCYA2 (MCP1) *in situ* at sites of injection, leading to chemotaxis of lymphocytes and monocytes in the lesions of skin reaction [47-49]. We found that IFNβ aberrantly regulated the levels of expression of several cytochrome P450 (CYP) enzymes (see Additional files 3,4,5,6). Type I IFN reduces the activity of CYP enzymes that metabolize various endogenous and exogenous substrates, probably leading to an increase in the potential for IFN-related hepatotoxicity [50].

Finally, the list of IRGs included various apoptosis regulators and HSP family members. ISRE-like sequences are identified in the regulatory element of CASP1, CASP4, CASP8, TNFRSF6 (FAS), TNFSF6 (FASL) and TNFSF10 (TRAIL), suggesting that IFNβ acts as a proapototic cytokine [51,52]. A recent study showed that early and sustained induction of TRAIL provides a marker for IFNβ treatment response in MS [53]. Furthermore, IFNβ-inducible apoptosis regulators play an immunoregulatory role. TNFR1-associated via death domain (TRADD) inhibits IFNγ-induced STAT1α activation [54]. Receptor-interacting serine-theronine kinase 1 (RIPK1) regulates TLR3-independent viral double-stranded RNA-induced type I IFN production [55]. Because HSPs in general act as an anti-apoptotic defender, the induction of HSP gene expression might occur as a counterbalance against upregulation of proapoptotic regulators. Alternatively, IRGs could directly enhance HSP expression. IFNβ-induced STAT1, by interacting with heat shock factor-1 (HSF1), activates the HSP70 and HSP90β gene promoters [56].

Conclusion

Microarray analysis showed that IFNβ immediately induces a burst of gene expression of proinflammatory chemokines and cytokines *in vitro* that have potential relevance to IFNβ-related early adverse effects in MS patients *in vivo*.

Abbreviations

MS = multiple sclerosis; IFNβ = interferon-beta; IRGs = IFNβ-responsive genes; PBMC = peripheral blood mononuclear cells; CNS = central nervous system; IFNγ = interferon-gamma; Th1 = T helper type 1; MHC = major histocompatibility complex; RRMS = relapsing-remitting multiple sclerosis; ISG = IFN stimulated gene; TLR = Toll-

like receptor; STAT = signal transducer and activator of transcription; IRF = interferon regulatory factor; ISRE = interferon-stimulated response element; IRE = interferon regulatory factor-recognition element; HSPs = heat shock proteins; CSF = cerebrospinal fluid; GPCR = G protein-coupled receptor; RGS = regulator of G protein signaling.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

JS, YN and HT carried out DNA microarray and real-time RT-PCR analysis, and JS drafted the manuscript. TY participated in the design of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

Additional material

Additional File 1

The gene list of cDNA microarray utilized in the present study. The complete gene list of cDNA microarray utilized in the present study is shown. It includes 1,258 well-annotated genes, selected from cytokines, growth factors and their receptors, apoptosis regulators, oncogenes, transcription factors, cell cycle regulators and housekeeping genes.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2377-6-18-S1.xls]

Additional File 2

Scatter plots of three distinct microarray experiments. The figure represents a scatter plot exhibiting the comparison between the fluorescence intensity (FI) of Cy5 signals in the longitudinal axis and FI of Cy3 signals in the horizontal axis. (a) the subject #1 (a 46 year-old healthy man), (b) the subject #2 (a 28 year-old healthy man), and (c) the subject #4 (a 27 year-old woman with RRMS who was a dropout of IFNβ treatment due to induction of frequent severe relapses).
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[http://www.biomedcentral.com/content/supplementary/1471-2377-6-18-S2.ppt]

Additional File 3

The complete list of upregulated genes in PBMC following exposure to IFNβ for 3 hours. Upregulated genes in PBMC of the subject #1 (a 46 year-old healthy man) by a 3 hour-exposure to 50 ng/ml recombinant human IFNβ are listed with Cy5/Cy3 signal intensity ratio, gene symbol, GenBank accession number, and gene name. In vivo IRG in T cells and non-T cells of RRMS patients reported previously (Ref. 16) are underlined.
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Additional File 4

The complete list of downregulated genes in PBMC following exposure to IFNβ for 3 hours. Downregulated genes in PBMC of the subject #1 (a 46 year-old healthy man) by a 3 hour-exposure to 50 ng/ml recombinant human IFNβ are listed with Cy5/Cy3 signal intensity ratio, gene symbol, GenBank accession number, and gene name.
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Additional File 5

The complete list of upregulated genes in PBMC following exposure to IFNβ for 24 hours. Upregulated genes in PBMC of the subject #1 (a 46 year-old healthy man) by a 24 hour-exposure to 50 ng/ml recombinant human IFNβ are listed with Cy5/Cy3 signal intensity ratio, gene symbol, GenBank accession number, and gene name. In vivo IRG in T cells and non-T cells of RRMS patients reported previously (Ref. 16) are underlined.
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Additional File 6

The complete list of downregulated genes in PBMC following exposure to IFNβ for 24 hours. Downregulated genes in PBMC of the subject #1 (a 46 year-old healthy man) by a 24 hour-exposure to 50 ng/ml recombinant human IFNβ are listed with Cy5/Cy3 signal intensity ratio, gene symbol, GenBank accession number, and gene name.
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Additional File 7

Top 20 upregulated genes in PBMC following exposure to IFNβ for 3 hours: two additional subjects. Upregulated genes in PBMC of the subject #2 (a 28 year-old healthy man) and #4 (a 27 year-old woman with RRMS who was a dropout of IFNβ treatment due to induction of frequent severe relapses) following a 3 hour-exposure to 50 ng/ml recombinant human IFNβ are listed with Cy5/Cy3 signal intensity ratio, gene symbol, and gene name. Both CXCR3 ligand (yellow) and CCR2 ligand (blue) chemokines are highlighted.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2377-6-18-S7.xls]

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Human astrocytes express 14-3-3 sigma in response to oxidative and DNA-damaging stresses

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Abstract

The 14-3-3 protein family consists of seven isoforms, most of which are expressed abundantly in neurons and glial cells, although the σ isoform, a p53 target gene originally identified as an epithelium-specific marker, has not been identified in the human central nervous system. Here, we show that human astrocytes in culture expressed 14-3-3σ under stress conditions. By Western blot, the expression of 14-3-3σ, p53 and p21 was coordinately upregulated in astrocytes following exposure to hydrogen peroxide, 4-hydroxy-2-nonenal (4-HNE) or etoposide, a topoisomerase II inhibitor. 14-3-3σ was induced by treatment with 5-aza-2′-deoxycytidine, suggesting a hypermethylated status of the gene promoter in astrocytes. In vivo, a small subset of hypertrophic reactive astrocytes, often showing a multinucleated morphology, expressed 14-3-3σ in active demyelinating lesions of multiple sclerosis (MS) and ischemic lesions of cerebral infarction, where the expression of 4-HNE and 8-hydroxy-2′-deoxyguanosine was enhanced in reactive astrocytes. Microarray analysis of etoposide-treated astrocytes verified upregulation of p53-responsive genes and concurrent downregulation of mitotic checkpoint-regulatory genes. These observations suggest that 14-3-3σ might serve as a marker of oxidative and DNA-damaging stresses inducing the mitotic checkpoint dysfunction in reactive astrocytes under pathological conditions.

Keywords: Astrocytes; DNA damage; 14-3-3 Sigma; Multiple sclerosis; Multinucleated morphology; p53

1. Introduction

The 14-3-3 protein family consists of evolutionarily conserved, acidic 30-kDa proteins composed of seven isoforms named β, γ, ε, ζ, η, θ and σ in mammalian cells. A homodimeric or heterodimeric complex composed of the same or distinct isoforms constitutes a large cup-like structure possessing an amphipathic groove with two ligand-binding capacity (Fu et al., 2000). The dimeric complex acts as a molecular adaptor that interacts in a phosphorylation-dependent manner with key signaling molecules involved in cell differentiation, proliferation, transformation and apoptosis. The 14-3-3 protein regulates the function of target proteins by restricting their subcellular location, bridging them to modulate catalytic activity and protecting them from dephosphorylation or proteolysis, although 14-3-3 has some isoform-specific functions (MacKintosh, 2004). The 14-3-3 protein is expressed most abundantly in neurons in the central nervous system (CNS), where it represents 1% of total cytosolic proteins. Aberrant expression and impaired function of 14-3-3 in the CNS are associated with pathogenetic mechanisms of Creutzfeldt-Jacob disease, Alzheimer disease, Parkinson disease, spinocerebellar ataxia, amyotrophic lateral sclerosis and multiple sclerosis (MS) (Zerr et al., 1998; Chen et al., 2003; Satoh et al., 2004; review for Berg et al., 2002).

Oxidative stress-induced damage of nucleic acids, proteins and lipids plays a crucial role in the pathogenesis of neurodegenerative diseases, cerebral ischemia and MS, in

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which activated macrophages/microglia serve as a major source of reactive oxygen and nitrogen intermediates (Smith et al., 1999). The cellular response to DNA damage is controlled by the cell cycle checkpoint function. Upon DNA-damaging insults, the cells rapidly arrest at two distinct cell cycle checkpoints; at the transition from G1 to S phase (G1 checkpoint) or from G2 to M phase (G2 checkpoint) to inhibit DNA replication and mitosis, until damaged DNA is properly repaired (Zhou and Elledge, 2000).

The 14-3-3σ isoform is originally identified as an epithelium-specific marker named stratifin (Leffers et al., 1993). The regulatory region of 14-3-3σ gene has two sets of the consensus sequence for binding of p53, a central regulator of DNA damage responses called "guardian of the genome" (Hoh et al., 2002). 14-3-3σ acts as a key component of the G2 checkpoint machinery, while cyclin-dependent kinase inhibitor 1A (CDKN1A) named p21, a prototype p53-responsive gene, regulates both G1 and G2 checkpoints (Chan et al., 1999). Exposure of the cells to DNA-damaging agents results in p53-dependent induction of 14-3-3σ, which in turn arrests the cells in the G2/M phase of cell cycle to allow the repair of damaged DNA, by sequestering the cdc2-cyclin B1 complex in the cytoplasm (Hermeking et al., 1997; Chan et al., 1999). 14-3-3σ-deficient cells incapable of maintaining the cell-cycle arrest undergo cell death as they enter mitosis (Chan et al., 1999). Since 14-3-3σ acts as a negative regulator of the cell cycle, epigenetic silencing of the 14-3-3σ gene by hypermethylation of the CpG-rich promoter region causes malignant transformation of the cells (Ferguson et al., 2000). Furthermore, 14-3-3σ is regulated at a protein level by activating a proteasome-dependent mechanism (Urano et al., 2002). Downregulation of 14-3-3σ allows human epidermal keratinocytes to escape replicative senescence (Dellambra et al., 2000).

At present, biological and pathological roles of 14-3-3σ in the CNS remain unknown. A previous study showed that the σ isoform is undetectable in the human CNS (Nakajima et al., 2003). Another study indicates that it is expressed in Pick bodies and human hippocampal neurons (Umahara et al., 2004). Here, we show that human astrocytes in culture express 14-3-3σ in response to oxidative and DNA-damaging stresses that upregulate p53 and p21. In vivo, 14-3-3σ is identified in a small subset of multinucleated hypertrophic reactive astrocytes in demyelinating lesions of MS and ischemic lesions of cerebral infarction, associated with enhanced oxidative stress and DNA damage responses. Our observations suggest that 14-3-3σ serves as a marker of oxidative and DNA-damaging stresses causing the mitotic checkpoint dysfunction in reactive astrocytes.

2. Materials and methods

2.1. Human astrocytes in culture

Human astrocytes were established from neuronal progenitor (NP) cells in culture as described previously (Satoh et al., 2004). NP cells isolated from the brain of a human fetus at 18.5 weeks gestation were obtained from BioWhittaker (Walkersville, MD). NP cells plated on a polyethyleneimine-coated surface were incubated in DMEM/F-12 medium (Invitrogen, Carlsbad, CA) containing an insulin-transferrin-selenium (ITS) supplement (Invitrogen), 20 ng/ml recombinant human epidermal growth factor (Higeta, Tokyo, Japan), 20 ng/ml recombinant human basic fibroblast growth factor (Pepro-Tech EC, London, UK) and 10 ng/ml recombinant human leukemia inhibitory factor (Chemicon, Temecula, CA), according to the methods described previously (Carpenter et al., 1999). For the induction of astrocyte differentiation, NP cells were incubated for several weeks in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (feeding medium). This incubation induced vigorous proliferation and differentiation of astrocytes accompanied by a rapid reduction in non-astroglial cell types. Their purity exceeded 98% by glial fibrillary acidic protein (GFAP) immunocytochemistry. DNA sequencing analysis verified that the p53 sequence of human astrocytes was identical to that of wild-type p53 (not shown).

2.2. Exposure of astrocytes to oxidative and DNA-damaging stresses

To load oxidative stress, astrocytes were incubated for 24 h in the serum-free DMEM/F-12 medium containing 10 or 100 μM hydrogen peroxide, a prototype oxidative stress inducer or in the feeding medium containing 20 μM 4-hydroxy-2-nonenal (4-HNE; Merck, Tokyo, Japan) dissolved in ethanol, a potent inducer of intracellular peroxide production (Uchida et al., 1999). To load DNA-damaging stress, the cells were incubated for varying periods in the feeding medium containing 20 μM etoposide (Sigma, St. Louis, MO) dissolved in DMSO, a topoisomerase II inhibitor that generates double-stranded DNA brakes. In some experiments, the serum-free culture supernatant was harvested and concentrated at an 1/85 volume by centrifugation on a Centricon-10 filter (Millipore, Bedford, MA).

To determine the methylation status of the 14-3-3σ promoter, astrocytes were incubated for 72 h in the feeding medium containing 5 μM 5-aza-2′-deoxycytidine (aza-dC; Sigma) dissolved in 50% acetic acid, a DNA methyltransferase inhibitor (Ferguson et al., 2000). Then, sodium bisulfite-treated genomic DNA was processed for methylation-specific PCR (MSP) analysis using the CpGenome DNA modification kit (Chemicon International) with the methylation status-specific primer sets listed in Table 1. To examine proteasome-dependent proteolysis of 14-3-3σ, astrocytes were incubated for 4 or 24 h in the feeding medium containing 10 μM MG-132 (Merck-Calbiochem), a proteasome inhibitor (Urano et al., 2004).

2.3. Western blot analysis

To prepare total protein extract, the cells were homogenized in RIPA lysis buffer composed of 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS and a cocktail of protease inhibitors (Roche Diagnositics, Tokyo, Japan), followed by centrifugation at 12,000 rpm for 20 min at room temperature (RT). The supernatant was collected for separation on a 12% SDS-PAGE gel. The protein concentration was determined by a Bradford assay kit (BioRad, Hercules, CA). After gel electrophoresis, the protein was transferred onto nitrocellulose membranes and immunolabeled at RT overnight with the antibodies listed in Table 2. Then, the membranes were incubated at RT for 30 min with horseradish peroxidase (HRP)-conjugated anti-mouse, rabbit or goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The specific reaction was visualized by using a chemiluminescent substrate (Pierce, Rockford, IL). After the antibodies were stripped by incubating the membranes at 50 °C for 30 min in stripping buffer composed of 62.5 mM Tris–HCl, pH 6.7, 2% SDS and 100 mM 2-mercaptoethanol, the membranes were processed for relabeling several times with different antibodies.

2.4. Microarray analysis

Five μg of total RNA, isolated from the cells by using TRIZOL reagent (Invitrogen), was in vitro amplified once and then cRNA was processed for analyzing a Whole Human Genome Oligonucleotide Microarray (G4112A, 41,000 genes; Agilent Technologies, Palo Alto, CA). The complete gene list is available online (http://www.chem.agilent.com/). To identify the genes whose expression was affected by DNA damage in cultured human astrocytes, cRNA isolated from the cells incubated for 24 h in the feeding medium containing 20 μM etoposide was labeled with a fluorescent dye Cy5, while cRNA of the

Table 1
Primers utilized for PCR analysis

Genes	GenBank accession no.	Sense primers	Antisense primers
14-3-3σ promoter (methylated)	AF029081	5′tggtagtttttatgaaaggcgtc3′	5′cctctaaccgcccaccacg3′
14-3-3σ promoter (unmethylated)	AF029081	5′atggtagtttttatgaaaggtgtt3′	5′ccctctaaccacccaccaca3′
14-3-3σ mRNA	NM_006142	5′tgcgagacaacctgacactgtgga3′	5′catactagtcctctcggcagggt3′
p53 mRNA	NM_000546	5′ttggaactcaaggatgcccaggct3′	5′tcagtctgagtcaggcccttctgt3′
p21 mRNA	NM_078467	5′gcggcagaccagcatgacagattt3′	5′cagaagatgtagagcgggcctttg3′
GDF15 mRNA	NM_004864	5′gactgccactgcatatgagcagtc3′	5′tgtttgggcaggaatcgggtgtct3′
BIRC5 mRNA	NM_001168	5′ggctgcaccacttccagggtttat3′	5′cagaagcacctctggtgccacttt3′
ASPM mRNA	NM_018136	5′gcctctgatgtacgaagtaggtcc3′	5′ggaatgccaagcgtatccatcacc3′

The primer sets specific for methylated or unmethylated 14-3-3σ promoter were utilized for methylation-specific PCR analysis, while other primer sets were used for real-time PCR analysis. *Abbreviations*: GDF15, growth differentiation factor 15; BIRC5, bacuoviral IAP repeat-containing protein 5 (survivin); ASPM, abnormal spindle-like, microcephaly associated.

cells incubated for the same period in the medium supplemented with vehicle (DMSO) alone was labeled with Cy3. The array was hybridized at 60 °C for 17 h in the hybridization buffer containing equal amounts of Cy3- or Cy5-labeled cRNA. It was then scanned by the Agilent scanner (Agilent Technologies), and the data were analyzed by using the Feature Extraction software (Agilent Technologies). The fluorescence intensities (FI) of individual spots were quantified following global normalization between Cy3 and Cy5 signals, followed by Lowess normalization. The ratio of FI of Cy5 signal versus FI of Cy3 signal exceeding 2.0 was defined as significant upregulation, whereas the ratio smaller than 0.5 was considered as substantial downregulation.

2.5. Real-time RT-PCR analysis

DNase-treated total cellular RNA was processed for cDNA synthesis using oligo(dT)$_{12-18}$ primers and SuperScript II reverse transcriptase (Invitrogen). Then, cDNA was amplified by PCR in LightCycler ST300 (Roche Diagnostics) using SYBR Green I and the primer sets listed in Table 1. The levels of expression of target genes were standardized against those of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene detected in parallel in the identical cDNA samples. All the assays were performed in triplicate.

2.6. Human brain tissues

Ten micron-thick serial sections were prepared from autopsied brains of four MS patients and eight non-MS subjects. The tissues were fixed with 4% paraformaldehyde or 10% neutral formalin and embedded in paraffin. MS cases included a 29-year-old woman with secondary progressive MS (SPMS) who died of asphyxia, a 40-year-old woman with SPMS who died of respiratory failure, a 43-year-old woman with primary progressive MS (PPMS) who died of

Table 2
Primary antibodies utilized for immunohistochemistry and Western blot analysis

Antibodies (clones)	Suppliers	Code	Origin	Antigen specificity	Concentration used for immunohistochemistry	Concentration used for Western blotting
14-3-3 sigma	Immunobiological Laboratory	18642	Rabbit	14-3-3 sigma isoform	1 μg/ml	1 μg/ml
14-3-3 epsilon	Immunobiological Laboratory	18643	Rabbit	14-3-3 epsilon isoform	2 μg/ml	NA
p53 (DO-7)	Nichirei	413231	Mouse	Wild-type and mutant form of p53	Prediluted	Further diluted at 1:500
p21 (F-5)	Santa Cruz Biotechnology	sc-6246	Mouse	p21	0.4 μg/ml	0.2 μg/ml
4-HNE (HNEJ-2)	Japan Institute for the Control of Aging	MHN-20	Mouse	4-hydroxy-2-nonenal-modified proteins	2 μg/ml	NA
8-OHdG (N45.1)	Japan Institute for the Control of Aging	MOG-20P	Mouse	8-hydroxy-2′-deoxyguanosine	2 μg/ml	NA
GDF15	Santa Cruz Biotechnology	sc-10603	Goat	Intracellular proform of GDF15	2 μg/ml	0.1 μg/ml
GDF15	R & D Systems	AF957	Goat	Secreted mature form of GDF15	NA	0.1 μg/ml
GFAP	Dako	N1506	Rabbit	GFAP	Prediluted	NA
GFAP (GA5)	Nichirei	422261	Mouse	GFAP	Prediluted	NS
MBP	Dako	N1564	Rabbit	MBP	Prediluted	NA
NF (2F11)	Nichirei	412551	Mouse	Human 70-kDa and 200-kDa NF	Prediluted	NA
CD68 (KP1)	Dako	N1577	Mouse	CD68	Prediluted	NA
HSP60	Santa Cruz Biotechnology	sc-1052	Goat	HSP60	NA	0.1 μg/ml

Abbreviations: 4-HNE, 4-hydroxy-2-nonenal; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; GDF15, growth differentiation factor 15; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; NF, neurofilament; HSP60, 60-kDa heat shock protein; NA, not applied.

hyperglycemia and a 33-year-old man with SPMS who died of sepsis. Detailed clinical and neuroradiological profiles of MS patients were described elsewhere (Satoh et al., 2004). Non-MS neurological and psychiatric disease cases included a 47-year-old man with acute cerebral infarction who died of sepsis, an 84-year-old man with acute cerebral infarction who died of disseminated intravascular coagulation, a 62-year-old man with old cerebral infarction who died of pancreatic cancer, a 56-year-old man with old cerebral infarction who died of myocardial infarction, a 36-year-old woman with schizophrenia who died of lung tuberculosis and a 61-year-old man with schizophrenia who died of asphyxia. Neurologically normal control cases included a 75-year-old woman who died of breast cancer and a 74-year-old woman who died of gastric and hepatic cancers. Autopsies on all subjects were performed at the National Center Hospital for Mental, Nervous and Muscular Disorders, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan. Written informed consent was obtained form all the cases. The present study was approved by the Ethics Committee of NCNP.

2.7. Immunohistochemistry

After deparaffination, tissue sections were heated in 10 mM citrate sodium buffer, pH 6.0, either by microwave at 95 °C for 10 min or by autoclave at 125 °C for 30 s in a temperature-controlled pressure chamber (Dako, Tokyo, Japan). They were treated at RT for 15 min with 3% hydrogen peroxide-containing methanol to block the endogenous peroxidase activity. The tissue sections were then incubated with phosphate-buffered saline (PBS) containing 10% normal goat serum or 10% normal rabbit serum at RT for 15 min to block non-specific staining. They were incubated in a moist chamber at 4 °C overnight with primary antibodies listed in Table 2. For 4-HNE and 8-hydroxy-2′-deoxyguanosine (8-OHdG) staining, the treatment with hydrogen peroxide-containing methanol was performed after immunolabeling with the primary antibodies to eliminate any potential effects caused by hydrogen peroxide-induced free radicals. The fine specificity of rabbit anti-14-3-3σ antibody (IBL, Gumma, Japan), mouse anti-4HNE monoclonal antibody (HNE-J2) and mouse anti-8OHdG monoclonal antibody (N45.1) was described elsewhere (Satoh et al., 2004; Toyokuni et al., 1995, 1997). After washing with PBS, the tissue sections were labeled at RT for 30 min with peroxidase-conjugated secondary antibodies (Nichirei, Tokyo, Japan), followed by incubation with a colorizing solution containing diaminobenzidine tetrahydrochloride (DAB) and a counterstain with hematoxylin. For negative controls, the tissue sections were incubated with a negative control reagent (Dako) instead of primary antibodies.

In some experiments, tissue sections were initially stained with mouse anti-GFAP monoclonal antibody (GA5, Nichirei), then followed by incubation with alkaline phosphatase-conjugated secondary antibody (Nichirei) and colorized with New Fuchsin substrate. After inactivation of the antibody by heating the sections by autoclave in 10 mM citrate sodium buffer, pH 6.0, they were relabeled with rabbit anti-14-3-3σ antibody (IBL), followed by incubation with peroxidase-conjugated secondary antibody (Nichirei) and colorized with DAB substrate.

3. Results

3.1. Human astrocytes in culture express 14-3-3σ in response to oxidative and DNA-damaging stresses

Cultured human astrocytes were exposed to hydrogen peroxide or 4-HNE, and then processed for Western blot analysis. Under baseline conditions, they expressed low levels of 14-3-3σ, p53 and p21 proteins (Fig. 1a and b, lane 1). The levels of 14-3-3σ, p53 and p21 were elevated markedly by a 24 h-exposure to 100 μM hydrogen peroxide or 20 μM 4-HNE (Fig. 1a, lane 3; Fig. 1b, lane 2), while the treatment with 10 μM hydrogen peroxide was ineffective (Fig. 1a, lane 2). Real-time RT-PCR analysis verified an oxidative stress-induced increase in 14-3-3σ and p21 mRNA levels (Fig. 2a, c and d). In contrast, an elevation of p53 protein levels in astrocytes by oxidative stress was not accompanied by a substantial increase of p53 mRNA levels, suggesting a major role of posttranscriptional regulation in p53 expression (Fig. 2b and e).

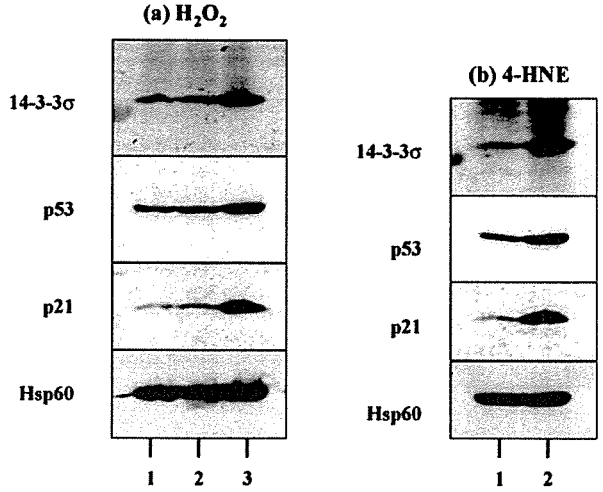

Fig. 1. The expression of 14-3-3σ, p53 and p21 in cultured human astrocytes following exposure to oxidative stress. Cultured human astrocytes were exposed for 24 h to two different oxidative stress-inducing stimuli. Then, they were studied by Western blot analysis of 14-3-3σ, p53, p21 and Hsp60 (a house-keeping gene product utilized as an internal control). The identical blot (150 μg of total cellular protein on each lane) was processed for sequential relabeling with different antibodies. (a) Hydrogen peroxide (H_2O_2) treatment. The lanes (1–3) indicate (1) untreated, (2) 10 μM hydrogen peroxide and (3) 100 μM hydrogen peroxide. (b) 4-Hydroxy-2-nonenal (4-HNE) treatment. The lanes (1 and 2) indicate treatment with (1) vehicle and (2) 20 μM 4-HNE.

Etoposide elevated greatly the levels of 14-3-3σ protein in cultured human astrocytes at 24 h after initiation of the treatment, while the elevation of p53 and p21 protein levels occurred much earlier, beginning at 4 h (Fig. 3a, lanes 1–6). Real-time RT-PCR analysis verified a great increase of 14-3-3σ mRNA but not of p53 mRNA in astrocytes exposed to etoposide (Fig. 2f and k). Treatment with aza-dC elevated markedly the protein levels of 14-3-3σ but not of p53 (Fig. 3b, lanes 1 and 2). Real-time RT-PCR analysis confirmed upregulation of 14-3-3σ mRNA in aza-dC-treated astrocytes (Fig. 2l). Methylation-specific PCR (MSP) analysis showed that an unmethylated DNA-specific PCR product was detected only when the cells were treated with aza-dC (Fig. 3d, lane 5). These observations indicate the constitutive hypermethylation of the 14-3-3σ promoter in astrocytes. MG-132 elevated the protein levels of p53 but not of 14-3-3σ, excluding a primary role of proteasome-dependent proteolysis in regulation of 14-3-3σ expression in cultured human astrocytes (Fig. 3c, lanes 1–3).

3.2. Multinucleated hypertrophic reactive astrocytes express 14-3-3σ in demyelinating lesions of MS and ischemic lesions of cerebral infarction

To identify astrocytes expressing 14-3-3σ in vivo in the human CNS, the brain, spinal cord and optic nerve sections of