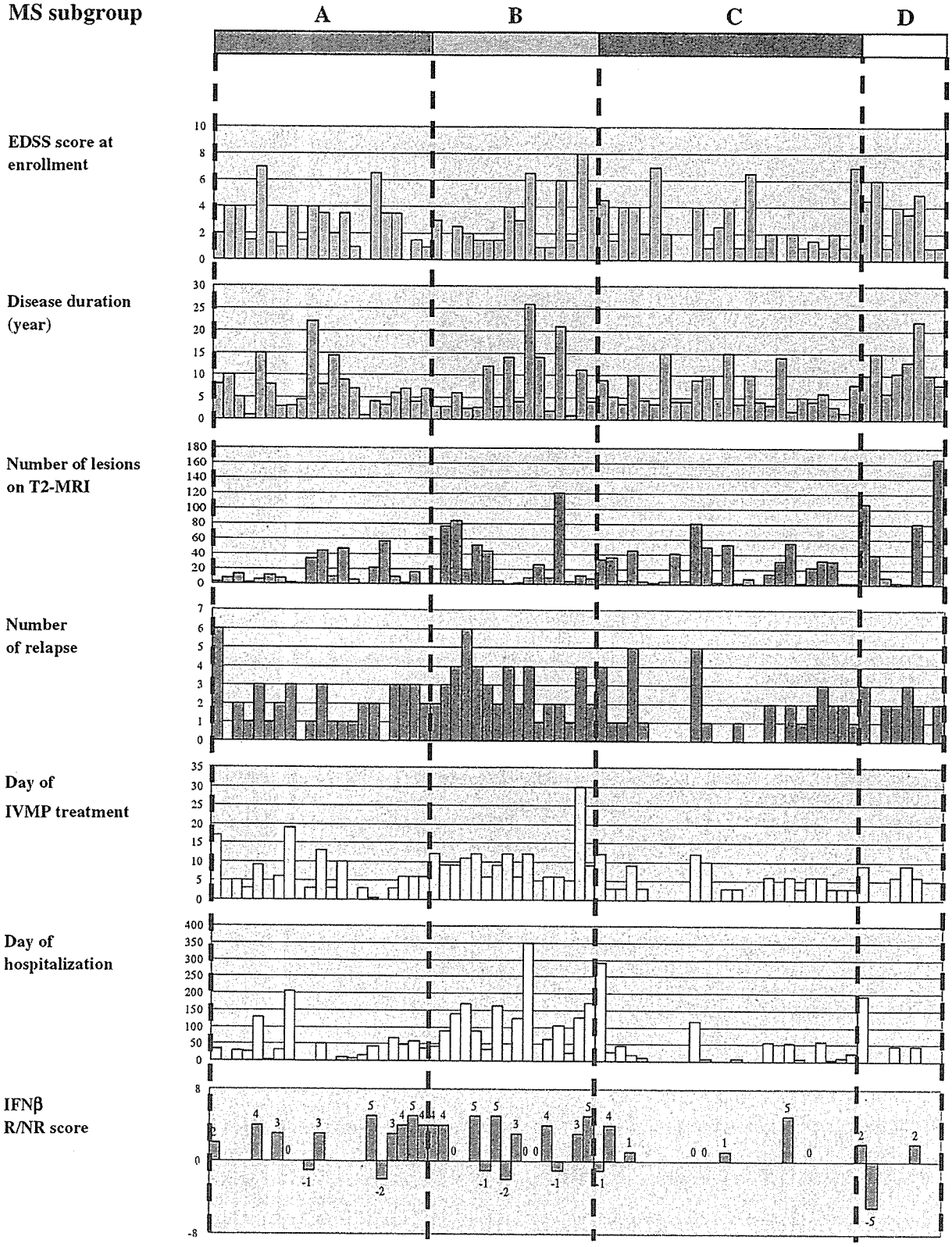
MS subgroup
A
B
C
D
EDSS score at enrollment
Disease duration (year)
Number of lesions on T2-MRI
Number of relapse
Day of IVMP treatment
Day of hospitalization
IFNβ R/NR score

Table 2
The therapeutic response to IFNβ in microarray-determined four MS subgroups

	Total	A	B	C	D	UC
IFNβ-treated patients (*n*)	46	14	14	11	5	2
Age of IFNβ-treated patients (average, SD)	34.9±9.2	33.2±7.6	36.5±10.4	33.1±8.3	36.2±13.3	41.5
Male to female ratio of IFNβ-treated patients	8 to 38	1 to 13	3 to 11	3 to 8	0 to 5	1 to 1
IFNβ responder/nonresponder score (average, SD)	1.9±2.6	2.5±2.3	2.1±2.6	1.3±2.1	−0.3±4.0	3
Dropout during a follow-up (*n*)	7	2	0	3	2	0
IFNβ responder (*n*, %)	19 (41.3%)	8 (57.1%)	8 (57.1%)	2 (18.2%)	0 (0%)	1 (50%)
IFNβ nonresponder (*n*)	7	2	3	1	1	0
Undetermined group (*n*)	13	2	3	5	2	1
The patients with IFNβ-related adverse effects (*n*, %)	29 (63.0%)	8 (57.1%)	9 (64.3%)	7 (63.6%)	4 (80%)	1 (50%)
Increase in the number of lesions on T2-weighted MRI during a follow-up (average, SD)	1.7±9.7	−2.0±7.1	2.8±6.6	7.6±15.8	−0.7±8.1	−3.5
The patients satisfied with IFNβ treatment (*n*, %)	17 (37.0%)	8 (57.1%)	6 (42.9%)	2 (18.2%)	0 (0%)	1 (50%)
The patients neither satisfied nor unsatisfied with IFNβ treatment (*n*)	21	4	7	7	2	1
The patients unsatisfied with IFNβ treatment (*n*)	8	2	1	2	3	0

Among 72 MS patients, 46 patients were treated with IFNβ for two years after enrollment. The therapeutic response was evaluated by IFNβ responder/nonresponder score shown in Table 1. Abbreviations: UC, unclassifiable.

3.4. Clinical characteristics of microarray-determined MS subgroups

Next, we investigated clinical characteristics of four MS subgroups (Supplementary Table 2 online and Fig. 3). No statistically significant differences were found among the subgroups in the age, disease duration, EDSS score, and the number of lesions on T2-weighted MRI at enrollment. However, there was a trend that the subgroup D showed a greater EDSS score and had a larger number of MRI lesions, suggestive of an advanced stage of the disease (Supplementary Table 2 online). The female outnumbered the male in all the subgroups. The male to female ratio was relatively higher in C, while no male patient was included in D. The patients with RRMS outnumbered those with SPMS in all the subgroups, although there was a mild bias for SPMS in B. The number of relapse, the day of IVMP treatment, and the day of hospitalization during preceding two years before enrollment were the largest and longest in subgroup B, and this difference was statistically significant, when compared between subgroups B and C ($p=0.0128$, 0.0183, and 0.0329 for each parameter) (Supplementary Table 2 online and Fig. 3). These observations indicate that the subgroup B included the patients who were the clinically most active before starting IFNβ.

In all MS subgroups, the conventional form of MS (CMS) greatly outnumbered non-CMS, the latter was composed of the opticospinal form (OSMS) and multifocal recurrent myelitis without optic nerve involvement. No obvious association was identified between a particular MS subgroup and the spinal cord involvement. However, 5 of 6 patients having the lesions restricted to the cerebrum (CBR) were included in subgroup C (Supplementary Table 2 online). These observations suggest that the status of T-cell gene expression might affect the lesion distribution in this subgroup.

3.5. IFN β responders were clustered in subgroups A and B

Based on the patient's own determination at enrollment, 72 MS patients were separated into two groups: 46 who started to receive IFNβ treatment for following two years, and 26 who were followed up without IFNβ treatment for successive two years (Supplementary Table 3 online). All the IFNβ-treated patients were evaluated by the IFNβ responder/nonresponder score (Table 1) at the end of the two year-treatment. They were classified into 19 IFNβ responders, 7 nonresponders, 13 undetermined subjects, and 7 dropouts (Table 2). The difference in the score among the subgroups (A: 2.5±2.3; B: 2.1±2.6; C: 1.3±2.1; and D: −0.3±4.3) did not reach the level of statistical significance (Table 2). However, there existed a trend that IFNβ responders were clustered either in subgroup A or B. Because the subgroup A contains the greatest proportion of IFNβ responders (57.1%), the patients of A were judged as being the most IFNβ responsive (Table 2). All the responders of A expressed a satisfaction on IFNβ treatment. The patients of the subgroup B also showed a good response equivalent to A (57.1%), although the number of satisfied patients was smaller. In contrast, only 2 of 11 IFNβ-treated patients in subgroup C (18.2%) and none of the patients in subgroup D were judged as IFNβ responders. The patients of C showed a trend for great increase in the number of MRI lesions during IFNβ treatment, consistent with the poor response to IFNβ (Table 2). A battery of IFNβ treatment-related adverse effects, including skin reactions, flu-like symptoms, leukocytopenia, depression, and amenorrhea, were observed in more than 50% of IFNβ-treated patients in all the subgroups (Table 2). Seven patients of the IFNβ-treated group discontinued the treatment: five due to adverse effects, one due to a severe relapse, and another by a personal reason.

We also studied T-cell gene expression profile of IFNβ-treated MS patients at 3 or 6 months after starting the

treatment. Although hierarchical clustering analysis classified these patients into several subgroups, they did not match with the subgroup A, B, C, or D determined at pretreatment (data not shown). Furthermore, no significant association was identified between these new clusters and the response to IFNβ. These observations suggest that T-cell gene expression profiling at pretreatment is the most valuable to predict the clinical outcome, whereas the analysis after starting IFNβ treatment is less informative.

3.6. Temporal profile of IFN-responsive gene expression in the first six months discriminated responders and nonresponders

Finally, we investigated the temporal expression profile of the genes with IFN-responsive promoter elements named IFN-responsive genes (IRGs) following IFNβ treatment. As we previously reported (Koike et al., 2003), IFNβ treatment for 6 months enhanced the expression of a battery of IRGs in T cells (Fig. 4). A remarkable difference was found between IFNβ responders (R) and nonresponders (NR) in the kinetics of several IRGs, such as IFN-stimulated protein 15 (ISG15), small inducible cytokine A2 (SCYA2, CCL2, or MCP-1), TNF receptor subfamily member 1B (TNFRSF1B, TNFRp75), and IFNα-inducible protein 27 (IFI27) (Fig. 5). The IFNβ responders exhibited a pattern of persistent upregulation during 6 months of the treatment. In contrast, the nonresponders showed a seesaw pattern, i.e. higher upregulation at 3 months than the responders, followed by substantial downregulation at 6 months. The differences between R and NR in the kinetics of both TNFRSF1B and IFI27 levels from 3 to 6 months were statistically significant ($p=0.0092$ and 0.0307, respectively) (Fig. 5). These observations suggest that IFNβ nonresponders also well respond to IFNβ at 3 months, but they could not maintain the responsiveness until 6 months.

4. Discussion

To elucidate the molecular basis underlying clinicopathological variability of MS, we conducted a comprehensive study that combines T-cell gene expression profiling and clinical characteristics of Japanese MS patients. Hierarchical clustering analysis of 286 genes differentially expressed between 72 untreated MS patients and 22 CN subjects classified a clinically heterogeneous population of MS into four distinct subgroups, named A, B, C, and D, and identified five gene classes numbered #1 to #5. The class

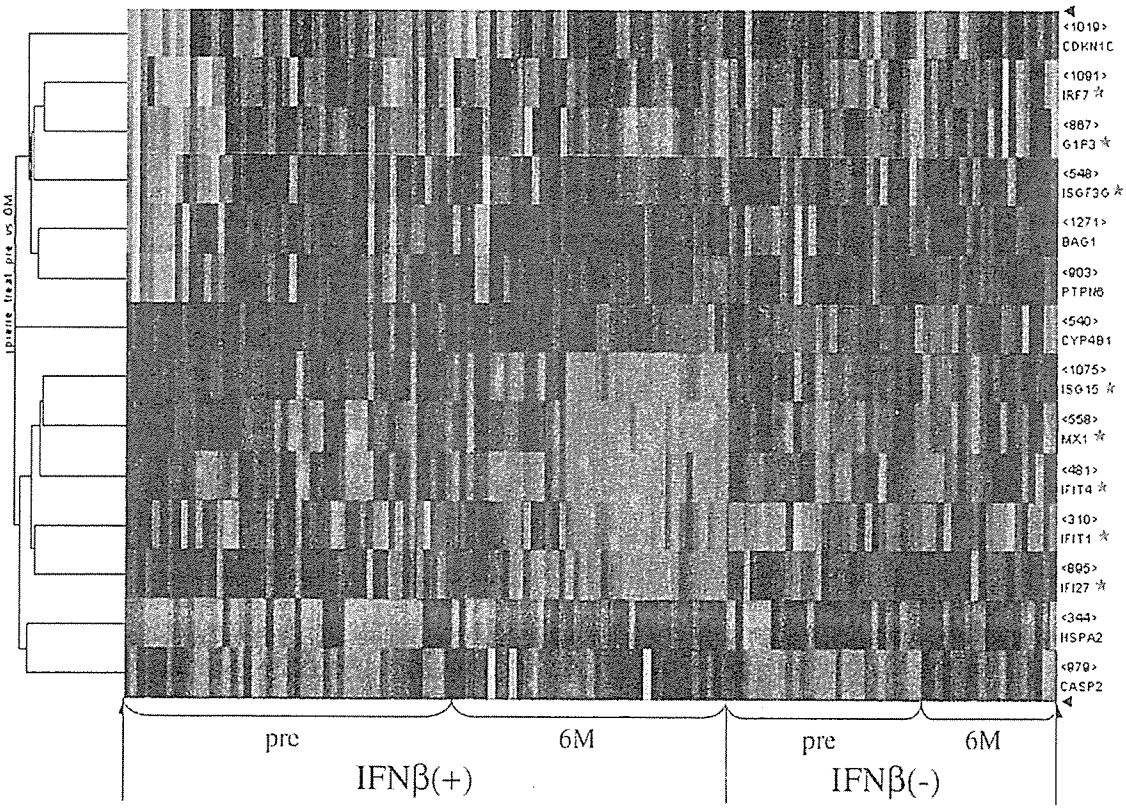

Fig. 4. Induction of IFN-responsive genes in IFNβ-treated MS patients. Seventy-two MS patients were divided into IFNβ-treated group (IFNβ+; $n=46$) and untreated group (IFNβ−; $n=26$). A cluster of known IFN-responsive genes (IRGs) indicated by the star were significantly upregulated exclusively in IFNβ-treated patients at 6 months after starting the treatment.

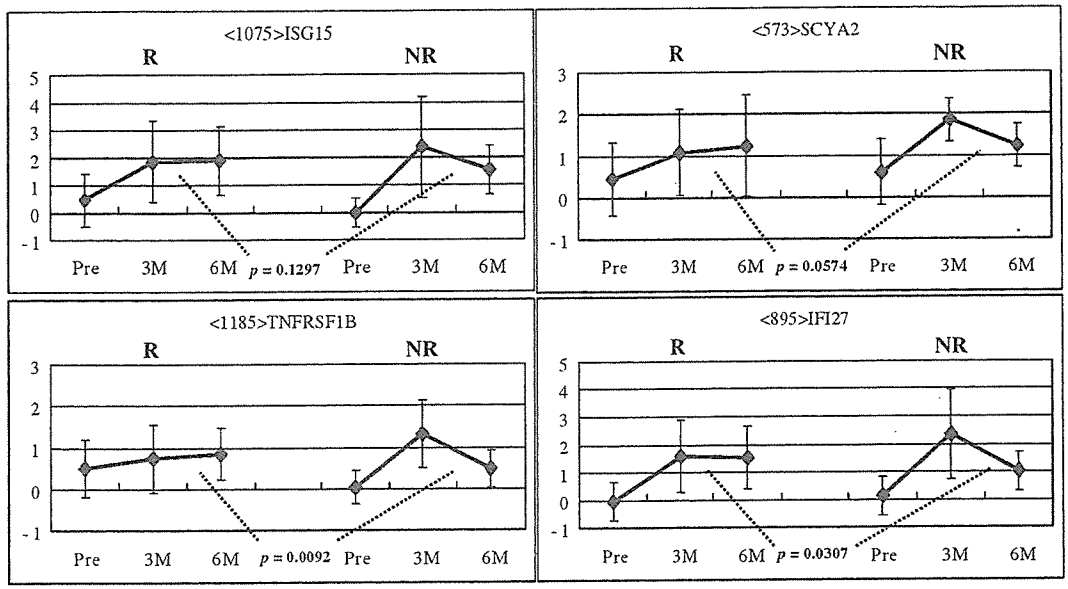

Fig. 5. Temporal profile of induction of IFN-responsive genes in IFNβ responders and nonresponders during IFNβ treatment. T-cell gene expression profiling was examined in 46 IFNβ-treated MS patients at three time points: before starting IFNβ treatment (Pre), and at 3 months (3M) and 6 months (6M) after starting the treatment. Based on the IFNβ responder/nonresponder score, they were separated into 19 IFNβ responders (R: the left) and 7 nonresponders (L: the right) as shown in Table 2. The temporal expression profile of IFN-responsive genes (IRGs), such as IFN-stimulated protein 15 (ISG15), small inducible cytokine A2 (SCYA2), TNF receptor subfamily member 1B (TNFRSF1B), and IFNα-inducible protein 27 (IFI27), is shown with the statistical differences in the kinetics during 3 to 6 months between R and NR. The vertical axis represents the gene expression level (GEL), while the horizontal axis indicates the time course.

#5 genes containing nine chemokines were upregulated exclusively in MS. The most clinically active subgroup B showed the highest upregulation of the class #5 genes. These observations suggest that the higher disease activity of B is in part attributable to overproduction of chemokines which promote lymphocyte and macrophage trafficking into the CNS (Balashow et al., 1999; Opdenakker et al., 2003). Recently, using this database as a training set for support vector machine (SVM) analysis of T cell gene expression, we found that the great majority of active RRMS patients were classified into MS subgroups, while clinically stable patients without obvious relapses for several years were occasionally classified into CN group (unpublished observations).

The class #5 genes upregulated in MS T cells include various cytokines, growth factors and their receptors, whose expression was detected at high levels in demyelinating lesions of MS, such as IL-12p40 (Windhagen et al., 1995), IL-10 (Hulshof et al., 2002), granulocyte colony-stimulating factor (GCSF) (Lock et al., 2002), platelet-derived growth factor receptor-alpha (PDGFRA) (Maeda et al., 2001), transforming growth factor-beta 2 (TGFB2) (Peress et al., 1996), and insulin-like growth factor-II (IGF-II) (Gveric et al., 1999). The class #5 genes also contain many apoptosis-signaling regulators pivotal for T cell development. It is worthy to note that nuclear receptor subfamily 4, group A, member 2 (NR4A2) in the class #5 was the most strongly upregulated gene in MS T cells. NR4A2 encodes an orphan member of the steroid–thyroid hormone receptor superfamily transcription factors designated Nurr1. Nurr1 is induced in T cells during apoptosis (Okabe et al., 1995) and the members of this family regulate clonal deletion of self-reactive T cells in the thymus (Zhou et al., 1996). Nurr1 activates the transcription of osteopontin (Lammi et al., 2004), a Th1 cytokine that plays a key role in progression of inflammatory demyelination in MS (Steinman and Zamvil, 2003). In contrast, a previous study showed that NR4A2 is downregulated in unfractionated PBMC of MS patients (Achiron et al., 2004), although the following study from the same group indicated a significant upregulation of NR4A2 in PBMC of both MS and SLE patients (Mandel et al., 2004). We validated upregulation of NR4A2 mRNA levels in MS T cells by quantitative real-time RT-PCR analysis (unpublished observations).

The present study suggests that the microarray-based classification of MS is useful to predict therapeutic response to IFNβ. The proportion of IFNβ responders greatly differed among MS subgroups: 57.1% in A, 57.1% in B, 18.2% in C and 0% in D. Furthermore, the responders are significantly different from the non-responders in the kinetics of IFN-responsive genes (IRGs). A panel of IRGs were upregulated persistently in IFNβ responders, whereas they were downregulated to some extent in nonresponders by 6 months. The precise reason for downregulation of IRGs in nonresponders after a long-term treatment remains unknown, because our study did not determine neutralizing antibody (NAb) development in individual patients. Unexpectedly, the patients of subgroup B exhibited a good response to IFNβ, despite its highest clinical activity. A recent study showed that IFNβ res-

ponders are characterized by higher relapse rates during the year prior to initiation of IFNβ treatment (Waubant et al., 2003), supporting our observations. In contrast, the patients of C with the poor response to IFNβ showed a trend for great increase in the number of MRI lesions during IFNβ treatment. A different study indicated that the number of on-treatment new T2 MRI lesions correlates with poor response to IFNβ-1a (Rudick et al., 2004), being consistent with our study. Although the subgroup D did not include any IFNβ responders, the number of MRI lesions did not increase much during the treatment, suggesting that this subgroup undergoes a neurodegenerative process independent of active inflammation (Steinman, 2001).

A number of differentially expressed genes between MS and CN include those having IFN-responsive elements in the promoter regions: p300 (EP300) and IFNα receptor 1 (IFNAR1) in the class #1, CXCL10, ATP-binding cassette (ABC) subfamily E member 1 (ABCE1 or RNS4I) (Bisbal et al., 1995), IFNγ-inducible protein 16 (IFI16), and STAT1 in the class #2, myxovirus resistance 2 (MX2) in the class #3, IFN-regulatory factors IRF9 and IRF2 in the class #4, and IFNα-16 (IFNA16) and CXCL9 (MIG) in the class #5. These observations suggest that T cells of MS patients have a constitutive defect in regulation of IFN signaling. We previously showed that CXCL9 expression was suppressed in T cells of 13 RRMS patients by a long-term treatment with IFNβ (Koike et al., 2003), suggesting that IFNβ produces a beneficial effect on MS by correcting the preexisting disturbance in the IFN-signaling pathway.

Finally, T-cell gene expression profiling identified an aberrant expression of key regulators for drug metabolism, whose role has not previously been proposed in MS (Nguyen et al., 2000). A panel of cytochrome P450 (CYP) family, which regulates Ca^{2+} influx in activated T cells (Aussel et al., 1994), were overexpressed in MS T cells. On the other hand, a wide range of ABC transporters in the classes #2, #3, and #4 were downregulated in MS T cells (see Supplementary Table 1 for all datasets). The ABC transporter superfamily regulates the transport of amino acids, ions, sugars, lipids and drugs across the cell membrane by consuming the energy derived from ATP hydrolysis. The downregulated genes in MS include ABCB1 and ABCG2 expressed on brain endothelial cells, which act as a main transporter in the blood–brain barrier and determine bioavailability of corticosteroids and mitoxantrone in the brain (Zhang et al., 2003). The clinicopathological relevance of opposing changes in CYP family enzymes and ABC transporters to MS remains to be further investigated.

In conclusion, T-cell gene expression profiling is highly valuable to identify distinct subgroups of MS associated with differential disease activity and therapeutic response to IFNβ. This approach could be applied for designing tailor-made treatment of MS.

Acknowledgements

This work was supported by grants from Research on Intractable Diseases, and Research on Psychiatric and Neurological Diseases and Mental Health, from the Ministry of Health, Labour and Welfare of Japan, Research on Health Sciences Focusing on Drug Innovation, the Japan Health Sciences Foundation (KH21101), and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), Japan. The authors thank Drs. Masaaki Niino and Hikoaki Fukaura, Department of Neurology, Hokkaido University Graduate School of Medicine, and Dr. Masanaga Yamawaki, Department of Neurology, Tokyo Medical and Dental University for their invaluable help.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneuroim.2006.02.004.

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T Helper Type 2-Biased Natural Killer Cell Phenotype in Patients with Pemphigus Vulgaris

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Pemphigus vulgaris (PV) is an autoantibody-mediated bullous disease, but the role of natural killer (NK) cells in its pathogenic process has never been examined in detail. Circulating $CD56^+CD3^-$ NK cells as well as $CD69^+$-activated NK cells were increased in PV patients compared with healthy controls and patients with other autoantibody-mediated autoimmune diseases, including immune thrombocytopenic purpura and myasthenia gravis. Gene expression analysis of highly purified NK cells demonstrated an increased expression of *IL-10* and decreased expression of *IL-12Rβ2*, *perforin*, and *granzyme B ex vivo* in PV patients *versus* healthy controls. The NK cells from PV patients also showed impaired signal transducer and activator of transduction4 phosphorylation upon *in vitro* IL-12 stimulation. Moreover, NK cells from PV patients exhibited reduced IL-10 production in response to *in vitro* stimulation with IL-2/IL-12. Finally, *IL-5* expression in NK cells was exclusively detected *ex vivo* in PV patients with active disease, and was lost in subsequent analyses performed during disease remission. Together these findings suggest that NK cells contribute to a T helper type 2-biased immune response in PV patients through impaired IL-12 signaling and an upregulation of IL-10 and IL-5.

Journal of Investigative Dermatology (2007) **127**, 324–330. doi:10.1038/sj.jid.5700527; published online 31 August 2006

INTRODUCTION

Pemphigus vulgaris (PV) is an autoimmune bullous disease mediated by anti-desmoglein 3 (Dsg3) autoantibodies that are capable of directly causing acantholysis in the epidermis (Amagai *et al.*, 1991, 1992; Tsunoda *et al.*, 2003; Payne *et al.*, 2005). The production of anti-Dsg3 autoantibodies in PV patients was shown to result from a functional collaboration between Dsg3-specific B and T cells (Nishifuji *et al.*, 2000; Veldman *et al.*, 2004b), but recent accumulating evidence indicates that regulatory mechanisms play an important role in controlling the pathogenic autoimmune response (Kronenberg and Rudensky, 2005). Potential cell types that modulate the autoimmune response include regulatory T cells, natural killer (NK) cells, and natural killer T cells. In this regard, Dsg3-specific T regulatory type 1 cells have the capacity to inhibit Dsg3-specific T helper type 2 (Th2) cells *in vitro* (Veldman *et al.*, 2004a). On the other hand, the roles of NK and natural killer T cells in the pathogenesis of PV have not been evaluated to date. NK cells have long been regarded as an essential component of innate immunity, because they exert nonspecific cytotoxic activity against virus-infected cells and tumor cells (Robertson and Ritz, 1990). However, recent studies have revealed that NK cells are also the source of various cytokines, including both Th1 and Th2 cytokines (Warren *et al.*, 1995; Mehrotra *et al.*, 1998; Hoshino *et al.*, 1999), and they control the adaptive immune response by interacting directly with dendritic cells, T cells, and B cells (Blanca *et al.*, 2001; Mailliard *et al.*, 2003; Zingoni *et al.*, 2004). Natural killer T cells are also capable of producing large amounts of both Th1 and Th2 cytokines upon stimulation and thereby controlling the acquired immune response (Van Kaer, 2005).

In this study, we investigated the involvement of these cell types and their potential regulatory functions in the autoimmune pathogenesis of PV, and found a unique Th2-biased property of NK cells.

RESULTS

Increased and activated circulating NK cells in PV patients

Flow cytometric analysis was performed to examine the proportions of lymphocyte subsets, including those with potential regulatory functions, such as naturally occurring $CD4^+CD25^{high}$ regulatory T cells, $CD56^+CD3^+$ natural killer T cells, and $CD56^+CD3^-$ NK cells from 22 PV patients, including two with active disease, and from 28 healthy controls (Table 1). A statistically significant difference was detected only for the proportion of NK cells ($P=0.003$). A representative flow cytometric finding for NK cells is shown

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ITP, immune thrombocytopenic purpura; MG, myasthenia gravis; NK, natural killer; PV, pemphigus vulgaris; Stat, signal transducer and activator of transduction; Th, T helper

Received 31 March 2006; revised 19 June 2006; accepted 12 July 2006; published online 31 August 2006

Table 1. Proportion of lymphocyte subsets in peripheral blood from PV patients and healthy controls determined by flow cytometric analysis[1]

	PV (n=22)	Healthy controls (n=28)	P-value
$CD4^+$	37.6±14.5	37.5±11.9	NS
$CD8^+$	30.4±11.2	27.0±11.0	NS
$CD19^+$	7.5±4.7	9.2±4.0	NS
$CD4^+CD25^{high}$ [2]	2.1±1.1	1.7±0.8	NS
$CD56^+CD3^+$	6.1±7.8	3.4±3.5	NS
$CD56^+CD3^-$	21.3±13.4	10.8±7.0	0.003

PV, pemphigus vulgaris; NS, not significant.
[1]Results are shown as the mean±SD (%). Comparisons were made by the Mann–Whitney *U*-test.
[2]Sample numbers of PV patients and healthy controls were 18 and 27, respectively.

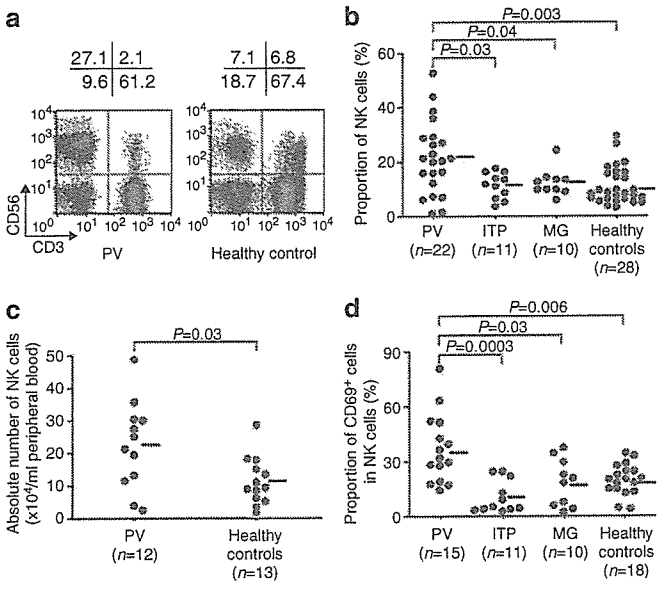

Figure 1. Analysis of NK cells in the peripheral blood from PV patients, ITP patients, MG patients, and healthy controls, by flow cytometry.
(**a**) Representative density plot analysis for evaluating the expression of CD56 and CD3 in a PV patient and healthy control. The cells in the lymphocyte fraction, gated on forward and side scatter, were evaluated. The numbers indicate the proportions of cells in the corresponding quadrants in the lower part. The upper left portion ($CD56^+CD3^-$) corresponds to NK cells. (**b**) The proportion of $CD56^+CD3^-$ NK cells in the circulating lymphocytes of 22 PV patients (two with active disease), 11 ITP patients, 10 MG patients, and 28 healthy controls. (**c**) The absolute number of $CD56^+CD3^-$ NK cells in 1 ml of peripheral blood from 12 PV patients (one with active disease) and 13 healthy controls. (**d**) The proportion of $CD69^+$-activated NK cells in the total NK cells of 15 PV patients (one with active disease), 11 ITP patients, 10 MG patients, and 18 healthy controls. Comparisons were made by the Mann–Whitney *U*-test. Horizontal bars represent the mean values.

in Figure 1a. Additional analyses of samples from 11 immune thrombocytopenic purpura (ITP) patients and 10 myasthenia gravis (MG) patients revealed that the increase in NK cells was unique to PV (Figure 1b). The absolute number of circulating NK cells was also significantly increased in PV patients compared with healthy controls (P=0.03, Figure 1c). We repeatedly assessed the NK cell proportion at different time points (interval 34–161 days) in four PV patients and three healthy controls, and found that the standard deviation of two sequential measurements was <4.0%.

We next evaluated the activation status of NK cells by examining the expression of CD69, a cell-surface marker expressed on activated NK cells (Marzio *et al.*, 1999). The proportion of $CD69^+$-activated NK cells in PV patients was significantly increased compared with the proportion in ITP patients, MG patients, or healthy controls (Figure 1d). These findings together indicate that NK cells were activated and expanded in the peripheral blood of PV patients.

Lack of difference in NK activity between PV patients and healthy controls

We assessed the NK activity in the peripheral blood mononuclear cell fraction from 14 PV patients and 12 healthy controls. NK cells from all the subjects showed cytotoxicity in an effector-to-target ratio-dependent manner. There was no statistically significant difference in the NK activity between PV patients and healthy controls (21.6±14.3% *vs* 17.0±10.5%), even though the proportion and activation status of the NK cells were higher in PV patients compared with healthy controls.

Upregulated expression of the *IL-10* gene and downregulated expression of the *IL-12Rβ2*, *perforin*, and *granzyme B* genes in NK cells from PV patients

To identify genes whose expression levels in NK cells were different between PV patients and healthy controls, we first carried out semi-quantitative PCR to evaluate the mRNA expression levels of 14 genes potentially involved in the regulatory function of NK cells, in NK cell preparations from four PV patients in remission, and four healthy controls (Table S1). A significant difference in the gene expression level between these two groups was detected for *IL-10*, which was upregulated in PV patients and for the *IL-12 receptor β2* (*IL-12Rβ2*), *perforin*, and *granzyme B*, which were downregulated in PV patients, compared with healthy controls.

To confirm these findings, we used the TaqMan® PCR system to quantify the mRNA expression levels in a larger number of subjects: six PV patients with active disease, 13 PV patients in remission, and 11 healthy controls (Figure 2). The expression of *IL-10* was again significantly higher, and the expression of *IL-12Rβ2*, *perforin*, and *granzyme B* was significantly lower in PV patients in remission and with active disease, compared with healthy controls. There was no difference in the expression levels of these four genes between PV patients with active disease and those in remission.

Impaired IL-12 signaling in NK cells from PV patients

The decreased *IL-12Rβ2* gene expression in the NK cells from PV patients could potentially lead to impaired IL-12 signaling. To test this hypothesis, NK cells from five PV patients, including one with active disease, and five healthy controls were stimulated with IL-12 and examined for the

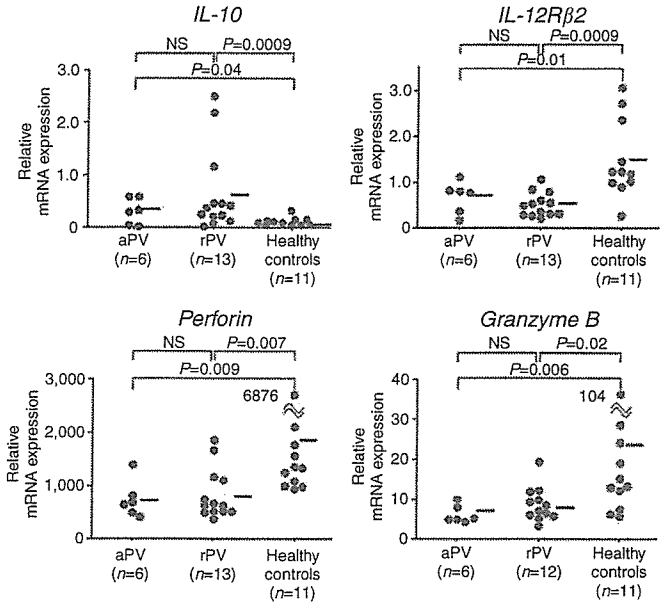

Figure 2. Relative mRNA expression levels of *IL-10*, *IL-12Rβ2*, *perforin*, and *granzyme B* in the NK cells from PV patients with active disease (aPV), PV patients in remission (rPV), and healthy controls. Total RNA was prepared from sorted $CD56^+CD3^-$ cells and subjected to Taqman® quantitative PCR. Individual mRNA expression levels were normalized to the mRNA expression level of *GAPDH*. Comparison was made by the Mann–Whitney *U*-test. Horizontal bars represent the mean values.

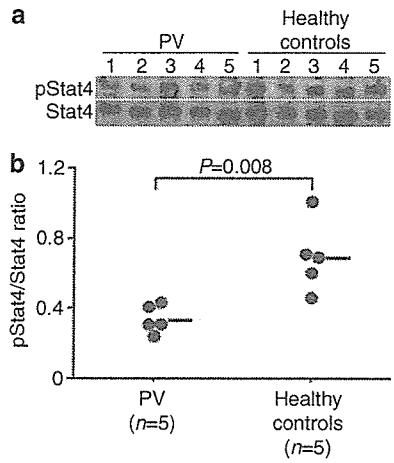

Figure 3. The degree of Stat4 phosphorylation in IL-12-stimulated NK cells in PV patients and healthy controls. (**a**) Sorted NK cells from five PV patients (one with active disease) and five healthy controls were stimulated with IL-12 *in vitro*, and subjected to immunoblot analysis to detect phosphorylated Stat4 (pStat4) and total Stat4. (**b**) The degree of Stat4 phosphorylation in five PV patients and five healthy controls. Comparison was made by the Mann–Whitney *U*-test. Horizontal bars represent the mean values.

phosphorylation of signal transducer and activator of transduction (Stat)4, one of the downstream events selectively induced by IL-12 signaling (Jacobson *et al.*, 1995). Immunoblotting of the cellular lysates of IL-12-stimulated NK cells to detect phosphorylated and total Stat4 revealed that the

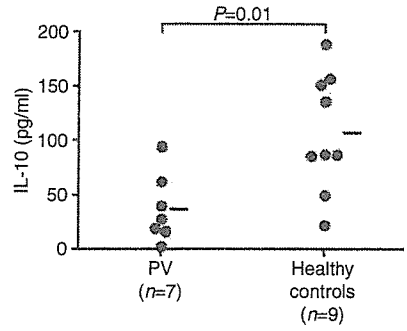

Figure 4. IL-10 production by NK cells from PV patients and healthy controls, upon *in vitro* stimulation with a combination of IL-2 and IL-12. NK cells freshly isolated from seven PV patients and nine healthy controls were cultured with IL-2 and IL-12 for 3 days, and the culture supernatants were subjected to ELISA for measurement of IL-10. Comparison was made by the Mann–Whitney *U*-test. Horizontal bars represent the mean values.

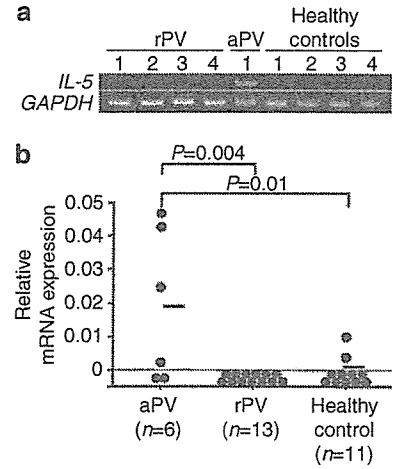

Figure 5. *IL-5* expression in NK cells from PV patients according to the disease activity. (**a**) *IL-5* and *GAPDH* expression by RT-PCR in sorted NK cells from four PV patients in remission, a PV patient with active disease, and four healthy controls. (**b**) *IL-5* mRNA expression levels determined using Taqman® quantitative PCR in six PV patients with active disease (aPV), 13 PV patients in remission (rPV), and 11 healthy controls. Each mRNA expression level was normalized to the *GAPDH* mRNA expression level. A relative mRNA expression level lower than 0 means the value was below the detectable limit. Comparison was made by the Mann–Whitney *U*-test. Horizontal bars represent the mean values.

degree of Stat4 phosphorylation was significantly lower in PV patients than in healthy controls ($P=0.008$; Figure 3).

Reduced IL-10 production from NK cells upon *in vitro* stimulation with IL-2 and IL-12 in PV patients

To confirm the upregulated IL-10 expression in NK cells from PV patients at a protein level, we measured IL-10 concentration in NK cell lysates using ELISA. However, IL-10 concentration was below the detection limit despite utilization of a highly sensitive commercial kit. NK cells were then stimulated with or without a combination of IL-2 and IL-12, a well-known stimulant for IL-10 production from NK cells

(Mehrotra *et al.*, 1998). IL-10 was undetectable without the stimulation. Interestingly, upon *in vitro* IL-2/IL-12 stimulation, NK cells from PV patients produced significantly less IL-10 than did those from healthy controls ($P=0.01$; Figure 4).

Upregulated *IL-5* gene expression in NK cells from PV patients with active disease

In patients with multiple sclerosis, *IL-5* expression by NK cells is upregulated in remission, but not in the active disease phase (Takahashi *et al.*, 2001). In contrast, we failed to detect *IL-5* mRNA in the PV patients in remission (Table S1). When the NK cells from additional PV patients were examined, *IL-5* mRNA expression was detected exclusively in the patient with active disease (Figure 5a). The *IL-5* expression by NK cells was further evaluated in 19 PV patients, including six with active disease, and 11 healthy controls using TaqMan® quantitative PCR (Figure 5b). *IL-5* mRNA expression was detected in four PV patients with active disease (67%), but in none of 13 PV patients in remission. Serial analysis in two PV patients with active disease revealed the loss of *IL-5* expression after the disease became quiescent.

DISCUSSION

We demonstrate here that NK cells are quantitatively and phenotypically altered in PV patients as follows: (i) an increased proportion and absolute number of NK cells in circulation; (ii) an increased proportion of $CD69^+$-activated cells; (iii) downregulated *IL-12Rβ2*, *perforin*, and *granzyme B* genes; (iv) impaired IL-12-induced Stat4 phosphorylation; (v) upregulated expression of *IL-10*; (vi) reduced IL-10 production upon *in vitro* IL-2/IL-12 stimulation; and (vii) upregulated expression of *IL-5* in association with active disease status.

The NK cells have been quantitatively analyzed in several autoimmune and inflammatory diseases. The majority of these diseases, including multiple sclerosis, Graves' disease, and psoriasis, are associated with a decreased proportion of NK cells (Kastrukoff *et al.*, 1998; Rojano *et al.*, 1998; Cameron *et al.*, 2003), but PV patients showed an increased proportion. In addition, the *in vivo* activation of NK cells appears to be unique to PV patients, because ITP patients and MG patients lacked this feature. Several lines of evidence indicate that NK cells control the fate of the autoimmune response. Specifically, the depletion of NK cells increases the severity of experimental autoimmune encephalomyelitis, a mouse model for multiple sclerosis, which is a well-documented Th1-mediated autoimmune disease (Zhang *et al.*, 1997). In contrast, NK-cell depletion delays the onset of disease and diminishes the severity of experimental autoimmune MG, a model for autoantibody-mediated autoimmune disease (Shi *et al.*, 2000). These findings indicate that regulatory roles of NK cells in the pathogenic immune response are different among the autoimmune diseases.

NK cells reside not only in the peripheral blood, but also in the spleen and lymph nodes (Ferlazzo *et al.*, 2004), where the interaction between autoreactive T and B cells primarily occurs (Kuwana *et al.*, 2002; Kent *et al.*, 2005). The recruitment of NK cells into the lymph nodes results in induction of the Th1 response by secretion of IFN-γ (Martin-Fontecha *et al.*, 2004). In PV patients, circulating NK cells showed characteristic gene expression profiles, irrespective of the disease status, that is, downregulated *IL-12Rβ2* and upregulated *IL-10*. Because IL-12Rβ2 is a key molecule in Th1 differentiation (Chan *et al.*, 1991; Yoshida *et al.*, 1994; Ma *et al.*, 1996), the downregulated *IL-12Rβ2* mRNA expression and impaired IL-12 signaling in NK cells observed in PV patients may lead to a reduced capacity to induce the Th1 response. Taken together with the upregulated expression of *IL-10* mRNA, it is likely that NK cells contribute to a Th2-biased environment in PV patients. As one of the effects of IL-10 on B cells is to promote production of antibodies, especially of the IgG4 isotype (Jeannin *et al.*, 1998), the upregulated IL-10 expression in NK cells may result in the IgG4-dominant anti-Dsg3 autoantibody response observed in PV patients irrespective of the disease status (Jones *et al.*, 1988; Shirakata *et al.*, 1990; Wilson *et al.*, 1993; Futei *et al.*, 2001).

In contrast to upregulated *IL-10* mRNA expression *ex vivo*, NK cells from PV patients produced less IL-10 than did those from healthy controls upon *in vitro* IL-2/IL-12 stimulation. This phenomenon might be explained by impaired IL-12 signaling observed in NK cells from PV patients. In this case, *in vivo* IL-10 upregulation by NK cells in PV patients is potentially mediated through IL-12-independent pathway. Alternatively, NK cells are already activated and exhausted *in vivo* in PV patients.

We also found an upregulated expression of *IL-5*, another Th2 cytokine, exclusively in PV patients with active disease. Interestingly, this pattern of *IL-5* expression in NK cells in association with the disease status is completely opposite to that seen in multiple sclerosis patients, in whom IL-5 is upregulated in remission, but downregulated in the active phase (Takahashi *et al.*, 2001). Distinct gene expression controls by the NK cells in PV and multiple sclerosis might reflect a difference in the role of Th1/Th2 balance in the disease pathogenesis. As IL-5-producing NK cells inhibit the Th1 response and drive Th2 polarization (Takahashi *et al.*, 2001), the expression of IL-5 by NK cells in patients with active PV further enhances the Th2-biased environment. This might also explain why IgE anti-Dsg3 antibodies are detected in PV patients with active disease, but not in those in remission (Spaeth *et al.*, 2001), because IL-5 is known to enhance IgE secretion (Pene *et al.*, 1988; Vercelli *et al.*, 1989).

In summary, our findings suggest that NK cells may be involved in the pathogenic autoimmune response in PV patients by promoting Th2 polarization, although additional studies using serial samples collected before and after the initiation of the treatment are necessary to confirm our theory.

MATERIALS AND METHODS

Patients and controls

We studied 28 patients (12 men) with PV that were diagnosed on the basis of all the following findings: (1) suprabasal acantholysis by histological examination; (2) IgG deposition on the keratinocyte cell surfaces by direct immunofluorescence; and (3) a positive test for serum IgG anti-Dsg3 antibody. The average age at examination was

51.0±10.8 years. Nine patients (32%) had additional IgG anti-Dsg1 antibody. At the time of blood sample collection, six patients had active disease, and the remaining 22 were in clinical remission, defined as having had no bullae or erosions during the past 2 months, irrespective of treatment.

We also examined 11 patients (two men) with ITP and 10 (two men) with MG to control for autoantibody-mediated autoimmune disease. The average age at examination of the ITP patients and MG patients was 53.7±19.2 and 53.5±18.4 years, respectively. The diagnoses of ITP and MG were based on the published criteria (Drachman, 1994; George *et al.*, 1996). Thirty-five healthy individuals (13 men) were also used as control subjects, and their average age at examination was 47.5±20.1 years. There was no statistically significant difference in sex distribution or age at examination between the PV patient group and the individual control groups. Twenty-two PV patients (76%), four ITP patients (36%), and six MG patients (60%) were taking low-dose prednisolone at the time of blood collection, and the mean daily dosage was 9.3 mg (range 1–19 mg), 8.1 mg (5–10 mg), and 5.8 mg (3–10 mg), respectively. This study was approved by the Keio University Institutional Review Board and conducted according to the Declaration of Helsinki Principles. Written informed consent was obtained from all the subjects.

Flow cytometric analysis

Peripheral blood mononuclear cells were incubated with fluorescence-conjugated mouse monoclonal antibodies, including anti-CD3-FITC, anti-CD4-FITC, anti-CD8-phycoerythrin, anti-CD56-phycoerythrin, anti-CD69-phycoerythrin-cyanin 5.1 (Beckman-Coulter, Hialeah, FL), and anti-CD19-Cy-Chrome (Becton Dickinson, San Diego, CA), followed by analysis with a FACSCalibur® flow cytometer (Becton Dickinson). All analyses were performed on the lymphocyte fraction gated based on the forward and side scatters. The absolute number of the cells of interest was quantified as the number per 1 ml of peripheral blood, based on the ratio to the number of FlowCount® microbeads (Beckman-Coulter). Appropriate fluorescence-labeled isotype-matched control antibodies were included in all the analyses.

NK activity

The nonspecific cytotoxic activity of NK cells was measured by the ^{51}Cr release assay according to a previously described method (Lanier *et al.*, 1983) with some modifications. Briefly, ^{51}Cr-labeled target cells (leukemia cell line K562) were mixed with freshly isolated peripheral blood mononuclear cells at effector-to-target ratios of 1:1, 5:1, 10:1, and 20:1 for 4 hours, and the radioactivity released into the culture supernatants was measured using an automatic gamma counter (Perkin-Elmer Life Sciences, Boston, MA). Each assay was performed in triplicate, and the percent lysis was calculated using the following formula: (experimental release −spontaneous release)/(maximal release in the presence of 1% Triton X−spontaneous release) × 100. NK activity was defined as the percent lysis at an effector-to-target ratio of 20:1.

Isolation of NK cells

NK cells were highly purified by a two-step sorting process using a MACS cell isolation system (Miltenyi Biotech, Bergisch Galdbach, Germany). Namely, $CD3^+$ and $CD14^+$ cells were depleted from peripheral blood mononuclear cells, then $CD56^+$ cells were positively selected. The proportion of $CD56^+CD3^-$ NK cells in the enriched fraction was 98.1±0.3%, as determined by flow cytometric analysis.

Analysis of mRNA expression

Sorted NK cells were used to determine semi-quantitatively their mRNA expression levels of 14 genes (listed in Table S2) that are potentially associated with NK cell function. In brief, total RNA was isolated using an RNeasy mini kit (Qiagen, Hilden, Germany), and treated with AMV RTase XL (TAKARA BIO, Otsu, Japan) in the presence of an oligo-dT primer to generate first-strand cDNA. The cDNA equivalent to 5 ng total RNA was subjected to PCR under the conditions shown in Table S2. The number of PCR cycles was adjusted to assure that the correlation between the amplified product and the amount of input cDNA fell within a linear range. The PCR products were then fractionated on agarose gels and visualized by ethidium bromide staining. The intensity of individual bands that corresponded to the expected molecular size was semi-quantified using NIH Image® available at http://rsb.info.nih.gov/nih-image/. The relative mRNA expression level was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The mRNA expression of the selected genes was further analyzed quantitatively using a Taqman® real-time PCR system with an ABI PRISM® 7900HT (Applied Biosystems, Foster City, CA). cDNA equivalent to 10 ng total RNA was subjected in duplicate to the reactions according to the manufacturer's protocol. Primer pairs and a TaqMan probe for *perforin*, *granzyme B*, *IL-5*, and *GAPDH* were purchased from Applied Biosystems, whereas those for *IL-10* and *IL-12Rβ2* were designed using Primer Express® Software v2.0 (Applied Biosystems) as follows: *IL-10*, sense (5′-GGCGCTGTCATCGATTTCTT-3′), antisense (5′-CTTGGAGCTTATTAAAGGCATTCTTC-3′), probe (5′-CAAGAGCAAGGCCGTGGAGCAGG-3′); and *IL-12Rβ2*, sense (5′-GGGCATTTTCTCAACGCATT-3′), antisense (5′-GCTGGATCTGGAATTTCTCTGCTA-3′), probe (5′-TTCTCCTAGCAGCCCTCAGACCTCAGTG-3′). The gene expression was standardized on the basis of serial dilutions of cDNA prepared from a healthy individual's peripheral blood mononuclear cells that were stimulated with phorbol 12-myrisate 13-acetate (25 ng/ml) and ionomycin (1 μg/ml) (Sigma, St Louis, MO). Relative expression levels were normalized to the expression level of *GAPDH*.

Evaluation of phosphorylated Stat4 in NK cells

The phosphorylation status of Stat4 in NK cells was evaluated as described previously (Toyoda *et al.*, 2004). Briefly, sorted NK cells were incubated with rIL-12 (50 ng/ml; R&D systems, Minneapolis, MN) at 37°C for 30 minutes. The soluble fraction derived from 10^5 cells was fractionated by SDS-7.5% polyacrylamide gel electrophoresis, and subjected to immunoblotting using the Can Get Signal system (TOYOBO, Osaka, Japan) with rabbit anti-phosphorylated Stat4 polyclonal antibodies (Zymed Laboratories, South San Francisco, CA) and peroxidase-conjugated anti-rabbit IgG antibodies (Cappel, Aurora, OH). The antibodies that bound to the membrane were subsequently visualized using Western Lightning™ Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA). After a stripping treatment with Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL), the membrane was re-subjected to immunoblotting using rabbit anti-Stat4 polyclonal antibodies (Santa

Cruz Biotechnology, Santa Cruz, CA). The intensity of individual bands was semi-quantitatively assessed using NIH Image®. The degree of phosphorylation was evaluated as the ratio of the intensity of phosphorylated Stat4 to that of total Stat4.

Quantification of IL-10 production by NK cells

To quantify IL-10 in freshly isolated NK cells, soluble fractions derived from NK cells (10^6 cells) were subjected to highly sensitive ELISA (Quantikine® HS; R&D systems) (sensitivity 0.5 pg/ml). To evaluate IL-10 produced by NK cells upon *in vitro* stimulation, NK cells (10^5/well) were cultured in 96-well flat-bottomed plates with or without a combination of rIL-12 (10 ng/ml) and rIL-2 (100 U/ml; Shionogi, Osaka, Japan) for 3 days. Culture supernatants were harvested and IL-10 concentrations were measured by BD OptEIA Human IL-10 ELISA (BD, Franklin Lakes, NJ) (sensitivity 2.0 pg/ml).

Statistical analysis

All comparisons for statistical differences between two patient groups were performed using the Fisher's two-tailed exact test or Mann–Whitney *U*-test as appropriate.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Dr Hidemi Toyoda (Mie University) for valuable comments on the evaluation of Stat4 phosphorylation. This study was supported by grants from the Ministry of Education, Sports, Science and Technology and the Ministry of Health, Labour, and Welfare.

SUPPLEMENTARY MATERIAL

Table S1. Results of semi-quantitative PCR for 14 immune-associated genes.

Table S2. Primer information.

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ARTHRITIS & RHEUMATISM
Vol. 54, No. 6, June 2006, pp 1946–1951
DOI 10.1002/art.21899

Increase in Circulating Endothelial Precursors by Atorvastatin in Patients With Systemic Sclerosis

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Objective. **To evaluate whether atorvastatin can increase bone marrow–derived circulating endothelial precursors (CEPs) and improve the vascular symptoms in patients with systemic sclerosis (SSc; scleroderma).**

Methods. **The study was designed as an open-label, prospective study involving 14 patients with SSc who received 10 mg/day of atorvastatin for 12 weeks and were followed up for the subsequent 4 weeks. CEPs were quantified at weeks 0 (pretreatment), 4, 8, 12 (during treatment), and 16 (posttreatment) by cell sorting followed by 3-color flow cytometry. Raynaud's phenomenon variables, global measures, and psychological scales as well as circulating angiogenic factors and endothelial activation/injury markers were serially assessed. The potential of CEPs to differentiate into mature endothelial cells was examined in cultures with angiogenic stimuli.**

Results. **None of the patients experienced an adverse event, but 1 dropped out because of an excessive decrease in serum total cholesterol. Atorvastatin treatment resulted in a 1.7- to 8.0-fold increase in CEPs from baseline levels ($P < 0.0001$), but the numbers returned to within baseline levels at posttreatment. However, 8 patients (62%) experienced a gradual decrease in the number of CEPs, even while taking atorvastatin. Variables indicating the extent of Raynaud's phenomenon improved significantly, and up-regulated levels of angiogenic factors and vascular endothelial activation/injury markers decreased significantly during atorvastatin treatment. These variables returned to within baseline levels after discontinuation of the drug. In contrast, atorvastatin failed to improve the in vitro maturation potential of CEPs.**

Conclusion. **The results of this pilot study suggest that atorvastatin treatment can increase CEPs and may be effective in improving Raynaud's phenomenon, even in SSc patients who have CEP dysfunction intrinsically.**

Systemic sclerosis (SSc; scleroderma) is a multiorgan disease characterized by excessive fibrosis and microvascular abnormalities (1). SSc vasculopathy mainly affects the small arteries and causes reduced blood flow and tissue ischemia, which can lead to Raynaud's phenomenon, digital ulcers, and gangrene (1). A primary mechanism for the vascular involvement in patients with SSc is thought to be the enhancement of vascular injury occurring as a result of the inflammation/immunopathologic process and the ischemia-reperfusion reaction. However, we have recently proposed another theory, that insufficient mechanisms of vascular repair, due to defective vasculogenesis, contribute to the pathogenic process (2).

Vasculogenesis requires the recruitment of bone marrow–derived circulating endothelial precursors (CEPs) to form the blood vessels (3). This process has been believed to occur exclusively during blood vessel development, but recent accumulating evidence indicates that CEPs contribute to vascular healing in response to vascular injury or ischemia in adults, by homing to the site of injury and then working in concert with existing mature endothelial cells (4,5). However, in healthy adults, CEPs are a very rare circulating cell population, being present in only ~1 of 10^6 leukocytes (2,4).

Human CEPs can be identified by a characteristic

Supported by grants from the Japanese Ministry of Health, Labor, and Welfare and the Japanese Ministry of Education, Science, Sports and Culture.

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Submitted for publication October 12, 2005; accepted in revised form March 3, 2006.

surface phenotype that is positive for CD34, CD133, and vascular endothelial growth factor (VEGF) receptor type 2 (VEGFR-2) (4). We have developed assay systems to evaluate the absolute number of CEPs and their maturation potential, and used them to examine the quantity and function of CEPs in patients with SSc (2). In these patients, we observed a markedly reduced number of CEPs, and the CEPs had an impaired maturation potential in response to angiogenic factors, in comparison with healthy controls.

This finding led us to propose that strategies to increase the levels of CEPs have therapeutic potential for SSc vasculopathy. One drug family with this potential is the 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase inhibitors, or statins. In addition to their lipid-lowering effect, statins have various potential activities, including the suppression of inflammation, improvement of vascular function, and inhibition of smooth muscle proliferation; these multiple functions are called pleiotropic effects (6). Statins have recently been shown to stimulate CEP kinetics and increase the number of CEPs in the circulation of patients with stable coronary artery disease (7). We therefore conducted the present open-label, prospective study to evaluate whether statins have the capacity to increase CEPs and improve vascular symptoms in patients with SSc.

PATIENTS AND METHODS

Study design. The present study, a single-center, open-label, prospective study, involved 14 patients with SSc at Keio University Hospital in Tokyo, Japan, conducted from June 2004 to March 2005. All patients fulfilled the American College of Rheumatology preliminary classification criteria for SSc (8). Patients were enrolled regardless of whether SSc was of the diffuse or limited cutaneous form, and regardless of the serum total cholesterol level. The exclusion criteria included age <18 years, pregnancy or lactation, a history of potential adverse effects associated with statins, current treatment with statins or drugs known to interact with statins (e.g., fibrates and cyclosporine), the presence of another well-defined rheumatic disease except Sjögren's syndrome, serious organ involvement (e.g., chronic respiratory failure due to pulmonary interstitial fibrosis or uncontrolled malabsorption), or other debilitating illness (e.g., cancer).

All of the study patients received atorvastatin at a dosage of 10 mg once a day for 12 weeks, and were then followed up for the subsequent 4 weeks. Atorvastatin was started in the fall (September–November) when the ambient temperature in Tokyo declines. Peripheral blood samples were obtained from all patients at 5 time points: week 0 (pretreatment), weeks 4, 8, and 12 (during treatment), and week 16 (posttreatment). All patients completed a daily diary to track the occurrence and rate the severity of Raynaud's phenomenon attacks at weeks 0, 4, 8, 12, and 16, and completed questionnaires for the assessment of functional status at weeks 0, 12, and 16. The patients were allowed to continue their other therapies during the study period, provided that the drug dosages were maintained at a constant level until the study was completed. The study protocol conformed to the ethics principles of the World Medical Association Declaration of Helsinki as reflected in a priori approval from our institutional review board, and written informed consent was obtained from each patient.

Measurement of functional status in relation to Raynaud's phenomenon. The activity, disability, pain, and psychological impact associated with Raynaud's phenomenon were evaluated using a proposed core set of outcome measures for studying Raynaud's phenomenon in SSc patients (9). These measures included the Raynaud's Condition Score (RCS; a daily self-assessment of Raynaud's phenomenon activity using a 0–10 ordinal scale), the Health Assessment Questionnaire Disability Index (scale 0–3) (10), visual analog scales (VAS) for Raynaud's phenomenon, digital ulcers, pain, and overall disease (scales of 0–3), a VAS for the physician's global assessment of health (scale 0–3), and the mood and tension scales of the Arthritis Impact Measurement Scales 2 (scale 0–10) (11). The RCS values over the 2-week period prior to each assessment visit were averaged and expressed as the mean ± SD.

Antinuclear antibody analysis. SSc-related antinuclear antibodies were identified using indirect immunofluorescence and immunoprecipitation assays, as described previously (12).

Quantification of CEPs. The absolute numbers of CEPs and overall proportion of CD34+ cells in 20 ml of peripheral blood were quantified as described previously (2). Briefly, samples partially enriched in CD34+ cells from peripheral blood mononuclear cells by a magnetic-activated cell sorter (MACS) immunomagnetic technique (Miltenyi Biotech, Bergisch Gladbach, Germany) were subjected to 3-color flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) with a fluorescein isothiocyanate (FITC)–conjugated anti-CD34 monoclonal antibody (mAb), phycoerythrin-conjugated anti-CD133 mAb (Miltenyi Biotech), and anti–VEGFR-2 mAb (Sigma, St. Louis, MO) in combination with biotin-conjugated goat anti-mouse IgG $F(ab')_2$ and streptavidin-PC5 (Beckman Coulter, Fullerton, CA). The expression of CD133 and VEGFR-2 was evaluated on gated CD34+ cells, and the total number of viable cells in the CD34+ cell–enriched fraction was determined by its ratio to the FlowCount microbeads (Beckman Coulter). All procedures were performed by the same operator (YO), who was blinded to the sample identity.

Circulating levels of angiogenic factors and endothelial activation/injury markers. The levels of VEGF, soluble vascular cell adhesion molecule 1 (VCAM-1), and soluble E-selectin in heparinized platelet-poor plasma and the levels of basic fibroblast growth factor (bFGF) in serum were measured using specific enzyme-linked immunosorbent assay kits (Quantikine; R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

In vitro maturation of CEPs. The potential of CEPs to differentiate into mature endothelial cells in response to angiogenic stimuli was evaluated as described previously (2). Briefly, CD133+ cells separated from peripheral blood mononuclear cells by MACS were coplated with CD133− cells on

fibronectin-coated chamber slides and cultured in endothelial cell basal medium 2 (EBM-2) supplemented with EBM-2 MV SingleQuots (Clonetics, San Diego, CA) for 5 days. The cells were fixed and incubated with mouse anti–VEGFR-2 or anti–von Willebrand factor (vWF) mAb (Dako, Carpinteria, CA) followed by incubation with AlexaFluor 568 mouse-specific IgG (Molecular Probes, Eugene, OR) and then with FITC-conjugated anti-CD45 mAb (Dako). The stained cells were examined with a confocal laser fluorescence microscope (LSM5 Pascal; Carl Zeiss, Göttingen, Germany). The proportion of mature endothelial cells derived from CEPs in this in vitro maturation process was calculated as the ratio of CD45−,vWF+ cells to CD45−,VEGFR-2+ cells, with results expressed as a percentage.

Statistical analysis. Frequencies between 2 groups were tested for statistical significance using Fisher's 2-tailed exact test. All continuous values are expressed as the mean ± SD. Changes in the absolute values at different time points from baseline (at week 0) were compared by repeated-measures analysis of variance. When the P value for this overall comparison was considered significant ($P < 0.05$), post-hoc pairwise comparisons were performed using Dunnett's test.

RESULTS

Baseline characteristics. The study group comprised 14 female patients with SSc, between the ages of 36 years and 75 years (mean ± SD age 57.4 ± 11.0 years). The disease duration from the first symptom attributable to SSc, including Raynaud's phenomenon, ranged from 13 months to 261 months (mean ± SD 121 ± 78 months). Seven patients each had diffuse SSc and limited cutaneous SSc. Assays for the antinuclear antibody status revealed 6 patients with anti–Scl-70/topoisomerase I, 5 with anticentromere, 2 with anti–RNA polymerase III, and 1 with anti–U1 RNP antibody. The modified Rodnan skin thickness score (13) ranged from 4 to 28 (mean ± SD 10.1 ± 8.0). All patients had Raynaud's phenomenon, and 2 had ulcers on multiple digital tips at the time of entry. Four patients were receiving low-dose prednisolone (<10 mg daily), but none of them were receiving an immunosuppressive agent or D-penicillamine. All patients were receiving at least 1 of the following vasoactive or antioxidant agents: oral prostacyclin, calcium channel blocker (e.g., amlodipine), low-dose aspirin, and vitamin E. Only 3 patients had hypercholesteremia (total cholesterol level >220 mg/dl).

Adverse events. All but 1 patient completed the 12 weeks of atorvastatin treatment and continued to receive stable doses of other medications throughout the

Table 1. Serial measurements of CEP numbers and other variables during atorvastatin therapy*

	Week 0 (pretreatment)	Week 4	Week 8	Week 12	Week 16 (posttreatment)	Overall P
CD34+ cells (× 10^3/20 ml peripheral blood)	7.8 ± 5.0	10.5 ± 7.3	11.2 ± 7.6	12.6 ± 9.2†	7.8 ± 4.7	0.03
CEP, /20 ml peripheral blood	132 ± 78	300 ± 188‡	295 ± 123‡	308 ± 208‡	111 ± 55	<0.0001
Raynaud's phenomenon variables						
Raynaud's Condition Score	4.41 ± 1.45	3.64 ± 1.46	3.63 ± 1.82	2.95 ± 1.60‡	3.39 ± 1.58	0.04
Patient's assessment by VAS	1.22 ± 0.73	NT	NT	0.73 ± 0.38†	1.03 ± 0.68	0.03
Psychological scales						
AIMS2 tension scale	2.46 ± 1.91	NT	NT	2.35 ± 1.73	2.49 ± 2.03	NS
AIMS2 mood scale	2.08 ± 1.51	NT	NT	1.69 ± 1.18	2.25 ± 1.86	NS
Disability and global measures						
HAQ DI	0.35 ± 0.29	NT	NT	0.35 ± 0.32	0.37 ± 0.31	NS
Patient's global assessment by VAS	1.10 ± 0.59	NT	NT	0.88 ± 0.73	0.91 ± 0.68	NS
Physician's global assessment by VAS	1.21 ± 0.52	NT	NT	0.89 ± 0.47	0.89 ± 0.47	NS
Pain scale, patient's assessment by VAS	0.55 ± 0.64	NT	NT	0.60 ± 0.68	0.51 ± 0.53	NS
Modified Rodnan total skin thickness score (scale 0–51)	8.8 ± 6.3	NT	NT	8.8 ± 6.4	8.6 ± 6.1	NS
Total cholesterol, mg/dl§	205 ± 36	155 ± 26‡	157 ± 23‡	158 ± 27‡	195 ± 26	<0.0001
Angiogenic factors						
VEGF, pg/ml	29.7 ± 9.1	29.6 ± 10.1	29.4 ± 8.7	25.8 ± 8.7†	26.3 ± 7.9	0.02
bFGF, pg/ml	14.3 ± 4.4	10.0 ± 5.4‡	10.1 ± 4.4‡	9.7 ± 5.7‡	12.8 ± 4.8	<0.0001
Endothelial activation/injury markers						
Soluble VCAM-1, ng/ml	594.9 ± 189.0	545.7 ± 192.7‡	496.5 ± 172.7‡	517.3 ± 169.5‡	556.1 ± 192.1‡	<0.0001
Soluble E-selectin, ng/ml	44.7 ± 16.0	42.2 ± 14.4	40.3 ± 13.2‡	42.9 ± 15.0	42.9 ± 14.6	0.007

* Values are the mean ± SD. CEP = circulating endothelial precursor; VAS = visual analog scale; NT = not tested; AIMS2 = Arthritis Impact Measurement Scales 2; NS = not significant; HAQ DI = Health Assessment Questionnaire Disability Index; VEGF = vascular endothelial growth factor; bFGF = basic fibroblast growth factor; VCAM-1 = vascular cell adhesion molecule 1.
† $P < 0.05$ versus week 0.
‡ $P < 0.01$ versus week 0.
§ One patient dropped out at week 8, due to an excessive decrease in the total cholesterol level to <100 mg/dl.

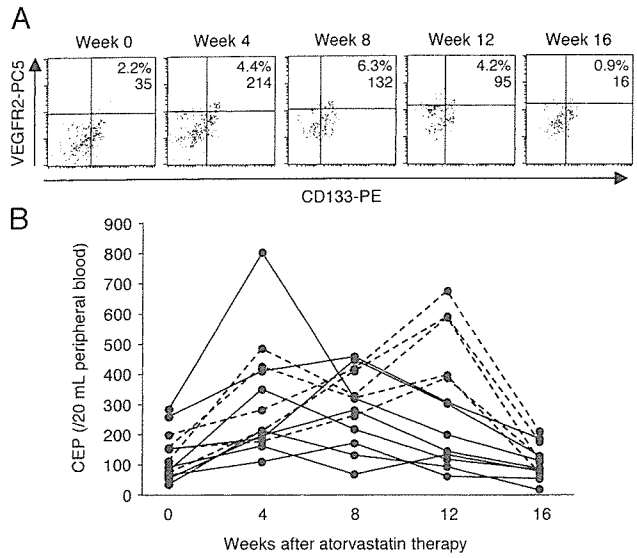

Figure 1. Effects of atorvastatin on the levels of circulating endothelial precursors (CEPs) in the peripheral blood of patients with systemic sclerosis (SSc). **A,** For serial measurements of CEPs by flow cytometry (shown in a representative SSc patient), the CD34+ cell–enriched fraction was stained with anti-CD34 (fluorescein isothiocyanate), anti-CD133 (phycoerythrin [PE]), and anti–vascular endothelial growth factor receptor type 2 (VEGFR-2) (VEGFR2-PC5) monoclonal antibody. CD34+ cells were gated and analyzed for the expression of CD133 and VEGFR-2. The upper-right section of the individual dot-plot images indicates the CD34+,CD133+,VEGFR-2+ CEPs. Values are the proportion of CEPs in the gated CD34+ cells and absolute number of CEPs in 20 ml of peripheral blood. **B,** Serial changes in absolute CEP numbers in 20 ml of peripheral blood were determined in peripheral blood samples from 13 SSc patients at 5 time points: week 0 (pretreatment), weeks 4, 8, and 12 (during atorvastatin treatment), and week 16 (posttreatment). CEP changes at the different time points from baseline were statistically significant ($P < 0.0001$) by repeated-measures analysis of variance, and CEP levels at weeks 4, 8, and 12 were significantly different from week 0 levels by post-hoc pairwise comparisons. The patients were divided into 2 groups based on the time course of the CEP response: solid lines indicate patients in group 1, in whom the number of CEPs peaked at week 4 or week 8 and decreased thereafter, even during atorvastatin treatment (n = 8), while broken lines indicate those in group 2, in whom the number of CEPs gradually increased during treatment (n = 5).

study. None of the patients experienced any adverse events during atorvastatin treatment, but 1 patient dropped out at week 8 because of an excessive decrease in total cholesterol level (to <100 mg/dl). After exclusion of this patient, the total cholesterol level in the remaining 13 patients with SSc was significantly reduced during atorvastatin treatment (reductions of 24%, 23%, and 23% at weeks 4, 8, and 12, respectively; $P < 0.0001$) but returned to within baseline levels at week 16 (Table 1).

Effects on CEPs. Serial flow cytometric analyses for CD133 and VEGFR-2 on gated CD34+ cells for a representative SSc patient are shown in Figure 1A. CEPs, identified as cells positive for both CD133 and VEGFR-2, were scarcely detected at pretreatment (week 0) and posttreatment (week 16), but were clearly visible during atorvastatin treatment (weeks 4, 8, and 12). The changes in the absolute number of CEPs before and after atorvastatin treatment are illustrated in Figure 1B. Treatment with atorvastatin resulted in a 1.7- to 8.0-fold increase (mean ± SD increase 3.8 ± 1.9) in the CEP number from baseline (mean ± SD 132 ± 78), but the CEP numbers (mean ± SD 300 ± 188, 295 ± 123, and 308 ± 208 at weeks 4, 8, and 12, respectively) did not reach the levels reported in healthy individuals (mean 1,074) (2). The CEP numbers returned to within baseline levels (mean ± SD 111 ± 55) after the cessation of atorvastatin at week 16 in all patients. The increase in CEP numbers from baseline at weeks 4, 8, and 12 was statistically significant ($P < 0.0001$) (Table 1).

The overall number of circulating CD34+ cells also increased during atorvastatin treatment ($P = 0.03$). Interestingly, there were 2 patterns of CEP response. In one pattern, CEP numbers peaked at week 4 or week 8 and were reduced thereafter, even during atorvastatin treatment (n = 8; group 1), and in the other response pattern, CEP numbers gradually increased during treatment (n = 5; group 2). When the clinical features were compared between these 2 groups, 75% of the patients in group 1 experienced digital ulcers at least once during the course of the disease, whereas none of the group 2 patients developed digital ulcers ever during the course of the disease ($P = 0.02$). Other characteristics distinguishing group 1, but without statistical significance, included a predominance of diffuse cutaneous SSc (63% versus 20% in group 2) and active digital ulcers at entry (25% versus 0% in group 2), but there was no difference in age at examination, disease duration, or RCS at entry between these 2 groups.

Effects on Raynaud's phenomenon activity. The serial changes in the Raynaud's phenomenon variables, psychological scores, disability and global measures, and pain scores during the study period are summarized in Table 1. The manifestations of Raynaud's phenomenon improved during atorvastatin treatment, with significant reductions in the RCS (−1.46 in mean daily score and 33% reduction in mean score at week 12; $P = 0.04$) and the patient's assessment by VAS (−0.49 in mean daily score and 40% reduction in mean score at week 12; $P = 0.03$). These variables tended to worsen after the discontinuation of atorvastatin. There was no difference in the

changes in these variables between CEP response groups 1 and 2. Although there were trends toward improvement in the mood scale and in the patient's and physician's global assessments by VAS at week 12, these changes were not statistically significant. None of the patients, including the 2 with digital ulcers at the time of entry, developed new digital ulcers during treatment with atorvastatin, but 1 patient developed new digital ulcers 2 weeks after the discontinuation of atorvastatin.

Effects on angiogenic factors and endothelial activation/injury markers. The circulating levels of angiogenic factors (VEGF and bFGF) and endothelial activation/injury markers (soluble VCAM-1 and soluble E-selectin) were measured before and after atorvastatin treatment (Table 1). The levels of these molecules are known to be elevated in the circulation of patients with SSc as compared with healthy controls (2,14). The levels of all of these circulating angiogenic factors and soluble endothelial activation/injury markers were significantly reduced during atorvastatin treatment as compared with baseline levels, but all returned to within baseline levels after the discontinuation of atorvastatin. There was no difference in the changes in these circulating markers between the 2 different CEP response groups.

Maturation potential of CEPs. The capacity of CEPs to maturate in response to in vitro angiogenic stimulation was evaluated at weeks 0 and 12 in 5 patients with SSc (2 in CEP response group 1, and 3 in group 2). The number of CEPs at week 12 was greater than that at week 0 in 4 of the patients, but was lower in 1 patient (in group 1). The maturation potential of CEPs was impaired in all 5 patients at baseline, and was not improved after the 12-week treatment with atorvastatin (mean ± SD 15.3 ± 4.9% at week 0 versus 17.9 ± 5.1% at week 12).

DISCUSSION

In this pilot study, short-term treatment with atorvastatin was associated with an increase in the number of CEPs in SSc patients. In addition, significant improvement in the ratings of Raynaud's phenomenon and reductions in the up-regulated levels of angiogenic factors and vascular endothelial activation/injury markers were observed during treatment with atorvastatin. Most of these variables returned to within baseline levels 4 weeks after the discontinuation of atorvastatin, confirming a link between these changes and atorvastatin therapy. The beneficial effects observed during atorvastatin treatment could be explained by the recruitment of CEPs into the periphery and the repair of injured endothelium, since statins have been shown to increase the number of CEPs and to promote vasculogenesis in vivo in animal models of ischemia (15–18). However, it is also possible that the observed clinical changes were mediated through other effects of statins, such as anti-inflammation mechanisms and the improvement of mature endothelial function (6).

A limitation of this study is that clinical assessment of Raynaud's phenomenon was carried out using the RCS and VAS rating in the setting of an open-label study; nevertheless, these measures are shown to be reliable for measuring Raynaud's phenomenon activity in SSc patients (9). This study would have benefited from inclusion of objective measures of small-vessel blood flow, such as pulse-wave analysis. Although the results of this preliminary study are encouraging, further multicenter, placebo-controlled trials involving a large number of SSc patients are necessary to confirm the clinical benefit of statins in SSc patients.

Despite their increase, the numbers of CEPs during atorvastatin treatment did not reach the level seen in healthy individuals, and the statin-induced CEP response was transient in many of the patients with SSc, especially those with antecedent digital ulcers. In addition, when a combination of angiogenic stimuli was used to induce CEP maturation, the statin failed to improve the impaired maturation potential of the CEPs. These observations indicate that although atorvastatin is certainly capable of improving CEP dysfunction in SSc patients, its effects are limited.

The mechanism causing the reduced numbers of CEPs in SSc patients is currently unknown, but the elevated level of circulating VEGF, which is known to be a critical mediator of CEP recruitment to the periphery (19), strongly suggests that CEPs and their stem cells in the bone marrow do not respond adequately to angiogenic stimuli, and VEGF and other angiogenic factors are up-regulated in compensation for this defect. The proposed mechanisms by which statins enhance the CEP numbers include increasing their proliferation and mobilization, and preventing CEP senescence and apoptosis within the bone marrow (15–18,20). The statins' ability to increase CEP numbers mimics the effects of VEGF, but is independent of the mechansim of action of VEGF (16).

It is therefore likely that CEPs and/or their stem cells in SSc patients are functionally altered and are intrinsically hyporesponsive to both VEGF and statins. In this regard, it would be interesting to investigate the phosphatidylinositol 3-kinase/Akt pathway in the CEPs from SSc patients, since the activation of this signaling

pathway is one of the critical events required for the increase in CEP levels induced by both VEGF and statins (15–17). Alternatively, continuous endothelial injury might lead to the eventual depletion of CEPs, as has been suggested to occur in patients with multiple risk factors for atherosclerosis (21). The transient CEP response induced by atorvastatin that was observed in patients with antecedent digital ulcers (group 1) might be consistent with this scenario.

Our study suggests that stimulation of CEP mobilization and/or differentiation may provide a novel therapeutic strategy for improving peripheral vascular disease in SSc patients. In this regard, in addition to statins, the administration of drugs that exert potent stimulatory effects on CEP kinetics, such as granulocyte–macrophage colony-stimulating factor (22) and granulocyte colony-stimulating factor (4), could augment vasculogenesis as a therapeutic intervention for ischemic complications in patients with SSc.

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Rheumatology 2006;45:851–854
Advance Access publication 17 January 2006

doi:10.1093/rheumatology/kel010

Concise Report

Two types of autoantibody-mediated thrombocytopenia in patients with systemic lupus erythematosus

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Objectives. **To determine whether autoantibodies to two platelet-specific antigens, glycoprotein IIb/IIIa (GPIIb/IIIa) and thrombopoietin receptor (TPOR), contribute to thrombocytopenia in patients with systemic lupus erythematosus (SLE).**
Methods. **Circulating B cells producing anti-GPIIb/IIIa antibodies and serum anti-TPOR antibodies were measured in 32 SLE patients with thrombocytopenia, 30 SLE patients without thrombocytopenia, 92 patients with idiopathic thrombocytopenia and 60 healthy controls. The megakaryocyte density in bone-marrow smears from all the patients with thrombocytopenia was evaluated.**
Results. **Anti-GPIIb/IIIa and anti-TPOR antibody responses were more frequent in SLE patients with thrombocytopenia than in those without thrombocytopenia (88 *vs* 17%, $P<0.0001$; and 22% *vs* 0%, $P=0.01$, respectively). The frequencies of these platelet-related antibodies were comparable between SLE patients with thrombocytopenia and patients with idiopathic thrombocytopenia. Twenty-nine (91%) SLE patients with thrombocytopenia had either anti-GPIIb/IIIa or anti-TPOR antibody, and six had both. In SLE patients with thrombocytopenia, the anti-TPOR-positive patients had significantly higher frequencies of megakaryocytic hypoplasia and poorer therapeutic responses to corticosteroids and intravenous immunoglobulin than did the anti-TPOR-negative patients, most of whom had the anti-GPIIb/IIIa antibody alone.**
Conclusions. **Anti-GPIIb/IIIa and anti-TPOR antibodies are major factors contributing to SLE-associated thrombocytopenia, but the clinical presentations associated with these autoantibodies are different.**

KEY WORDS: Autoantibodies, Glycoprotein IIb/IIIa, Systemic lupus erythematosus, Thrombocytopenia, Thrombopoietin receptor.

Thrombocytopenia is a major haematological complication in patients with systemic lupus erythematosus (SLE) [1]. The pathogenesis of thrombocytopenia in SLE patients is heterogeneous, but the most common mechanism is believed to be increased platelet clearance mediated by anti-platelet autoantibodies, which is analogous to the mechanism seen in patients with idiopathic thrombocytopenic purpura (ITP) [1]. Other potential mechanisms include thrombotic thrombocytopenic purpura, disseminated intravascular coagulation, haemophagocytic syndrome, antiphospholipid syndrome and impaired thrombopoiesis. Antiplatelet autoantibodies in ITP patients preferentially recognize platelet surface glycoproteins (GP), and the most common target is GPIIb/IIIa [2]. A recent study by Michel *et al.* [3] showed that anti-GPIIb/IIIa antibodies also play a primary role in SLE-associated thrombocytopenia. On the other hand, we recently identified autoantibodies to thrombopoietin receptor (TPOR), also called c-Mpl, which is clinically associated with thrombocytopenia in SLE patients and inhibits thrombopoietin (TPO)-dependent megakaryogenesis *in vitro* [4]. In this study, the roles of these two types of autoantibody responses in SLE-associated thrombocytopenia were evaluated.

Materials and methods

Patients and controls

We studied 32 patients with SLE who had thrombocytopenia (mean platelet count 23×10^9/l, range $5–57\times10^9$/l) and were followed at Keio University Hospital between 1997 and 2004. The inclusion criteria were as follows: (i) requirement for treatment because of a significant bleeding tendency; (ii) pretreatment bone marrow films were available; and (iii) exclusion of clinically apparent conditions that can cause thrombocytopenia, i.e. disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, haemophagocytic syndrome and drug-induced thrombocytopenia. The control subjects were 30 SLE patients who had never been thrombocytopenic and 60 healthy individuals. All the SLE patients satisfied the American College of Rheumatology preliminary criteria [5], and three with thrombocytopenia and four without thrombocytopenia additionally satisfied the Sapporo criteria for antiphospholipid syndrome [6]. We also examined 92 patients with idiopathic thrombocytopenia, defined as thrombocytopenia ($<100\times10^9$/l) that is not accompanied by morphological evidence of dysplasia in the bone marrow and cannot be attributed to other

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Received 17 October 2005; revised version accepted 8 December 2005.

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