Differential Expression of CD11c by Peripheral Blood NK Cells Reflects Temporal Activity of Multiple Sclerosis[1]

Toshimasa Aranami, Sachiko Miyake, and Takashi Yamamura[2]

Multiple sclerosis (MS) is an autoimmune disease, showing a great degree of variance in temporal disease activity. We have recently demonstrated that peripheral blood NK cells biased for secreting IL-5 (NK2 bias) are associated with the remission state of MS. In this study, we report that MS patients in remission differentially express CD11c on NK cell surface (operationally defined as $CD11c^{high}$ or $CD11c^{low}$). When we compared $CD11c^{high}$ or $CD11c^{low}$ patients, the expression of IL-5 and GATA-3 in NK cells supposed to endow a disease-protective NK2 phenotype was observed in $CD11c^{low}$ but not in $CD11c^{high}$ patients. In contrast, the $CD11c^{high}$ group showed a higher expression of HLA-DR on NK cells. In vitro studies demonstrated that NK cell stimulatory cytokines such as IL-15 would up-regulate CD11c expression on NK cells. Given previous evidence showing an association between an increased level of proinflammatory cytokines and temporal disease activity in MS, we postulate that inflammatory signals may play a role in inducing the $CD11c^{high}$ NK cell phenotype. Follow-up of a new cohort of patients showed that 6 of 10 $CD11c^{high}$ MS patients developed a clinical relapse within 120 days after evaluation, whereas only 2 of 13 $CD11c^{low}$ developed exacerbated disease ($p = 0.003$). As such, a higher expression of CD11c on NK cells may reflect the temporal activity of MS as well as a loss of regulatory NK2 phenotype, which may allow us to use it as a potential biomarker to monitor the immunological status of MS patients. ***The Journal of Immunology*, 2006, 177: 5659–5667.**

Multiple sclerosis (MS)[3] is a chronic inflammatory disease of the CNS, in which autoreactive T cells targeting CNS Ags are presumed to play a pathogenic role (1). A large majority of the patients with MS (~70%), known as relapsing-remitting MS, would develop acute exacerbations of disease between intervals of remission. It is currently believed that relapses are caused by T cell- and Ab-mediated inflammatory reactions to the self-CNS components, and could be controlled at least to some degree by anti-inflammatory therapeutics, immunosuppressants, or plasma exchange.

The clinical course of MS varies greatly among individuals, implicating difficulties to predict the future of each patient. For example, patients who had been clinically inactive in the early stage of illness could abruptly change into active MS accompanying frequent relapses and progressive worsening of neurological conditions. There are a number of unpredictable matters in MS, including an interval between relapses, responsiveness to remedy and the prognosis in terms of neurological disability. To provide better quality of management of the patients, searches of appropriate biomarkers are currently being warranted (2).

We have recently shown that surface phenotype and cytokine secretion pattern of peripheral blood NK cells may reflect the disease activity of MS (3, 4). A combination of quantitative PCR and flow cytometry analysis has revealed that NK cells in clinical remission of MS are characterized by a higher frequency of $CD95^{-}$ cells as well as a higher expression level of IL-5 than those of healthy subjects (HS) (3). As IL-5-producing NK cells, referred to as NK2 cells (5), could prohibit Th1 cell activation in vitro (3), we interpreted that the NK2 bias in MS may contribute to maintaining the remission state of MS. More recently, we have found that MS patients in remission can be further divided into $CD95^{high}$ and $CD95^{low}$, according to the frequency of $CD95^{+}$ cells among NK cells (4). Notably, memory T cells reactive to myelin basic protein, a major target Ag in MS, were increased in $CD95^{high}$ patients, compared with $CD95^{low}$. Of note, $CD95^{high}$ NK cells exhibited an ability to actively suppress the autoimmune T cells, whereas those from $CD95^{low}$ patients did not. These results suggest that NK cells may accommodate their function and phenotype to properly counterregulate autoimmune T cells in the remission state of MS.

Recently, a distinct population of NK cells that express CD11c, a prototypical dendritic cell (DC) marker, was identified in mice (6, 7). As the $CD11c^{+}$ NK cells exhibited both NK and DC functions, they are called as "bitypic NK/DC cells." CD11c associates with integrin CD18 to form CD11c/CD18 complex and is expressed on monocytes, granulocytes, DCs, and a subset of NK cells. Although precise functions are unclear, it has been reported that CD11c is involved in binding of iC3b (8), adhesion to stimulated endothelium (9) or phagocytosis of apoptotic cells (10). The initial purpose of this study was to evaluate CD11c expression and function of $CD11c^{+}$ NK cells in MS in the line of our research to characterize NK cells in MS. On initiating study, we noticed that there was no significant difference between MS and HS in the frequency of $CD11c^{+}$ NK cells. However, expression levels of CD11c were significantly higher in MS. We further noticed that up-regulation of CD11c is seen in some, but not all, patients with MS. So we have operationally classified MS into $CD11c^{low}$ and $CD11c^{high}$.

In this study, we demonstrate that IL-5, characteristic of NK2 cells (5), were significantly down-regulated in $CD11c^{high}$ than

Department of Immunology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan

Received for publication May 3, 2006. Accepted for publication July 28, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the Ministry of Health, Labor and Welfare of Japan and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation.

[2] Address correspondence and reprint requests to Dr. Takashi Yamamura, Department of Immunology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan. E-mail address: yamamura@ncnp.go.jp

[3] Abbreviations used in this paper: MS, multiple sclerosis; HS, healthy subject; DC, dendritic cell; MFI, mean fluorescence intensity; ECD, energy-coupled dye.

0022-1767/06/$02.00

$CD11c^{low}$ NK cells. In contrast, expression of HLA-DR class II molecule was up-regulated in $CD11c^{high}$ NK cells. Notably, both CD11c and HLA-DR on NK cells were reproducibly induced in vitro in the presence of IL-15 (11) or combination of inflammatory cytokines, known to be increased in the blood of MS (12–14). Furthermore, we found that the remission state of $CD11c^{high}$ is unstable in comparison to $CD11c^{low}$, as judged by an increased number of the patients who exacerbated during the 120 days after examining NK cell phenotypes. These results suggest that the $CD11c^{high}$ group of patients may be in more unstable condition than $CD11c^{low}$, presenting with reduced regulatory functions of NK cells.

Materials and Methods

Subjects

Twenty-five patients with relapsing-remitting MS (15) (male (M)/female (F) = 8/17; age = 37.7 ± 11.1 (year old)) and 10 sex- and age-matched HS (M/F = 3/7; age = 39.9 ± 12.2 (year old)) were enrolled for studying NK cell phenotypes. All the patients were in the state of remission at examination as judged by magnetic resonance imaging scanning and clinical assessment. They had not been given immunosuppressive medications, or corticosteroid for at least 1 mo before examination. They had relatively mild neurological disability (expanded disability status scale <4) and could walk to the hospital without any assistance during remission. The same neurologist followed up the patients regularly (every 3–4 wk) and judged the occurrence of relapse by using magnetic resonance imaging and clinical examinations. Information on NK cell phenotype or other immunological parameters was never given to either the neurologist or the patients at the time of evaluation. To precisely determine the onset of relapse, patients were allowed to take examination within a few days after a new symptom appeared. Written informed consent was obtained from all the patients and the Ethics Committee of the National Center of Neuroscience (NCNP) approved the study.

Reagents

Mouse IgG1 isotype control-PE, anti-CD3-energy-coupled dye (ECD), anti-CD4-PE, anti-CD8-PC5, anti-CD56-PC5, anti-CD69-PE, and anti-HLA-DR-FITC mAbs were purchased from Immunotech. Anti-CD11c-PE and anti-CD95-FITC were purchased from BD Pharmingen. Recombinant human cytokines were purchased from PeproTech. AIM-V (Invitrogen Life Technologies) was used for cell culture after supplementing 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen Life Technologies).

Cell preparation and NK cell purification

PBMC were separated by density gradient centrifugation with Ficoll-Hypaque PLUS (Amersham Biosciences). To purify NK cells, PBMC were treated with NK isolation kit II (Miltenyi Biotec) twice, according to the manufacturer's protocol. Briefly, PBMC were labeled with a mixture of biotin-conjugated mAbs reactive to non-NK cells and magnetic microbead-conjugated anti-biotin mAbs. The magnetically labeled non-NK cells were depleted with auto-MACS (Miltenyi Biotec) and this procedure always yielded >95% purity of NK cells when assessed by the proportions of $CD3^-CD56^+$ cells with flow cytometry.

Flow cytometry

To evaluate the expression of CD11c, CD95, or other surface molecules on NK cells, PBMC were stained with anti-CD3-ECD, anti-CD56-PC5, and FITC- or PE-conjugated mAbs against molecules of our interest and were analyzed with EPICS flow cytometry (Beckman Coulter). Mean fluorescence intensity (MFI) of CD11c was measured on gated $CD11c^+$ fraction or whole NK cells.

Stimulation of purified NK cells with proinflammatory cytokines

Purified NK cells (1×10^5/well) were stimulated in the presence or absence of IL-4, IL-8, IL-12, IL-15, IL-18, IL-23, TNF-α, and GM-CSF or combination of IL-12, IL-15, and IL-18 for 3 days. We analyzed CD11c expression after staining the cells with anti-CD11c-PE, anti-CD3-ECD, and anti-CD56-PC5. The concentration of IL-12 was at 10 ng/ml, and those of the other cytokines were at 100 ng/ml.

RT-PCR

Total RNA were extracted with a RNeasy Mini kit (Qiagen) from purified NK cells, and the cDNA were synthesized with Super Script III first strand systems (Invitrogen Life Technologies) according to the manufacturer's protocol. For quantitative analysis of IL-5, IFN-γ, GATA-3, and T-bet, the LightCycler quantitative PCR system (Roche Diagnostics) was used. Relative quantities of mRNA were evaluated after normalizing each expression levels with β-actin expression. PCR primers used were as follows: β-actin-sense, AGAGATGGCCACGGCTGCTT, and -antisense, ATTTGCGGTGGACGATGGAG; IFN-γ-sense, CAGGTCATTCAGATGTAGCG, and -antisense, GCTTTTCGAAGTCATCTCG; IL-5-sense, GCACACTGGAGAGTCAAACT, and -antisense, CACTCGGTGTTCATTACACC; GATA-3-sense, CTACGGAAACTCGGTCAGG, and -antisense, CTGGTACTTGAGGCACTCTT; T-bet-sense, GGAGGACACCGACTAATTTGGGA, and -antisense, AAGCAAGACGCAGCACCAGGTAA.

Statistical analysis of remission rate

We set the first episode of relapse after blood sampling as an end point, although we followed clinical course of each patient for up to 120 days, regardless of whether they developed relapses. No patients developed second relapse during the 120 days. When the neurologist prescribed corticosteroids without knowing any information on the NK cell phenotype, the patient was considered as the dropout at that time point. Remission rate was calculated as Kaplan-Meier survival rate, and statistical difference between $CD11c^{low}$ and $CD11c^{high}$ MS was evaluated with the log-rank test.

Results

CD11c on NK cells is up-regulated in MS remission

First, we confirmed that PBMC from healthy individuals and MS contain $CD11c^+$ NK cells (Fig. 1), which constitute a major population of whole NK cells. We then noticed that proportion of $CD11c^+$ NK cells as well as its levels of expression greatly varied among individuals, particularly in MS. To examine this issue further, we systemically examined 25 MS patients in remission and 10 HS for NK cell expression of CD11c. Whereas 20–80% of NK cells are $CD11c^-$ in HS (Fig. 1*c*), almost all NK cells were $CD11c^+$ in some MS patients (Fig. 1, *c* and *e*). However, reflecting a great degree of variance, comparison between HS and MS did not reveal a significant difference (Fig. 1*c*). In contrast, when we measured the MFI of CD11c expression on $CD11c^+$ NK cells, it was significantly higher in MS as compared with HS (Fig. 1*a*). This difference was also noticed when MFI of CD11c was measured for all the NK cell populations (Fig. 1*b*). It was interesting to know whether the levels of CD11c expression may correlate with NK cell functions. Therefore, we operationally divided the MS patients into $CD11c^{low}$ and $CD11c^{high}$ subgroups (Fig. 1*a*), by setting the border as (the average + 2 × SD) of the values for HS.

$CD11c^{high}$ NK cells express HLA-DR more brightly than $CD11c^{low}$ NK cells

It was previously reported that infection with certain viruses would accompany up-regulation of CD11c on NK cells (16). This raises a possibility that the increased expression of CD11c in $CD11c^{high}$ MS may reflect an activation state of NK cells caused by some sort of stimuli. To verify this hypothesis, we examined surface expression of cell activation markers (CD69 and HLA-DR). Although CD69, an early activation marker, was not detectable on NK cells (Fig. 2*a*), NK cells from MS, particularly $CD11c^{high}$ MS, significantly overexpressed HLA-DR on surface (Fig. 2). Interestingly, HLA-DR expression was also up-regulated on $CD4^+$ T cells from $CD11c^{high}$ MS compared with those from HS (data not shown). These results indicate that NK cells and T cells are differentially activated in $CD11c^{high}$ MS, $CD11c^{low}$ MS, and HS.

Absence of NK2 bias in $CD11c^{high}$ MS

We have previously reported that a higher level of IL-5 expression (NK2 bias) is one of the characteristics of NK cells of MS in

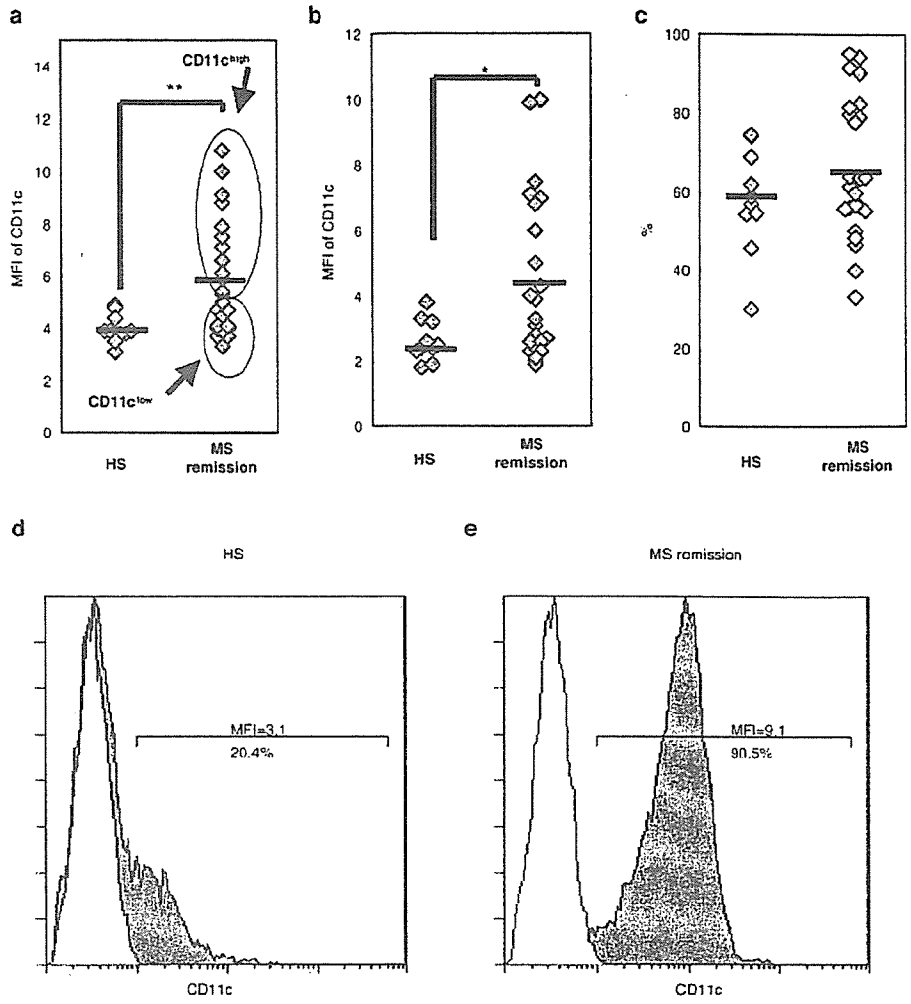

FIGURE 1. CD11c on NK cells is up-regulated in MS in remission. *a*, PBMC from HS (n = 10) and MS patients in remission (n = 25) were stained with anti-CD11c-PE, -CD3-ECD, and -CD56-PC5 mAb, and CD11c expression was measured on the $CD11c^+$ fraction gated within whole NK cells ($CD11c^+CD3^-CD56^+$ cells) as mean fluorescence intensity (MFI). Each dot represents the data from individual patients. $CD11c^{high}$ and $CD11c^{low}$ groups of patients are encircled as described in the text. *b*, In parallel, CD11c expression (MFI) was measured for the whole NK cells ($CD3^-CD56^+$ cells), which yielded a similar result. *c*, The proportions of $CD11c^+$ cells among whole NK cells are plotted. No significant difference was noted between HS and MS remission. *d* and *e*, Representative histogram patterns of CD11c on NK cells (closed histogram) from a single healthy subject (HS) (*d*) and a patient corresponding to $CD11c^{high}$ MS (*e*). Open histograms represent isotype control staining. Values represent proportions of $CD11c^+$ fraction (%) and MFI for $CD11c^+$ cells. Mann-Whitney U test was used for statistical analysis. Horizontal bars indicate the mean values. *, $p < 0.05$; **, $p < 0.01$.

remission (3). Although the mechanism for NK2 bias in MS remains to be further studied, up-regulation of GATA-3 has recently been reported in the induction of NK2 cells in mice (17). To explore the possible difference in the functions of $CD11c^{high}$ and $CD11c^{low}$ NK cells, we isolated NK cells from $CD11c^{high}$ or $CD11c^{low}$ group of patients and measured the mRNA levels of representative cytokines IFN-γ and IL-5 as well as corresponding transcription factors T-bet and GATA-3. As shown in Fig. 3, mRNA expression of both IL-5 and GATA-3 was significantly higher in $CD11c^{low}$ MS compared with HS or $CD11c^{high}$ MS, indicating that NK2 bias thought to be characteristic of MS remission is restricted to $CD11c^{low}$ MS. In contrast, there were no differences in mRNA expression of IFN-γ and T-bet among these three groups. Because NK cells from $CD11c^{high}$ patients expressed HLA-DR most brightly, we speculate that NK2 bias associated with $CD11c^{low}$ MS would attenuate when NK cells are further activated or differentiated.

NK cell stimulatory proinflammatory cytokines induce up-regulation of CD11c

We next attempted to explore the mechanism(s) for up-regulation of CD11c on NK cells in $CD11c^{high}$ MS. Because both NK cells and $CD4^+$ T cells overexpressed HLA-DR in $CD11c^{high}$, is it probable that immune signals influencing both innate and acquired immunity are operative. So we hypothesized that cytokine signals that have been implicated in the pathogenesis of MS may play a role. We cultured NK cells from HS in the presence or absence of cytokine(s) for 3 days, and evaluated the CD11c expression (MFI). We focused our attention to IL-12, IL-15, and IL-18, which are known to stimulate NK cells with or without help of other cytokines. Notably, they are reportedly elevated in the serum or blood lymphocytes of MS patients as compared with HS (11–14, 18, 19), and prior studies suggest that they may play an important role in autoimmune diseases (20–24). As shown in Fig. 4, although IL-12 and IL-18 showed only a marginal effect on purified NK cells, IL-15 consistently induced 2- to 3-fold up-regulation of CD11c compared with control culture without addition of cytokines. As IL-12 and IL-18 were reported to synergistically work in various settings (25, 26), we then examined whether combinations of these cytokines may induce CD11c. Combination of IL-15 and IL-12 or of IL-15 and IL-18 did not augment the CD11c expression to the level higher than that could be induced by IL-15 alone. However, the combination of IL-12 and IL-18 did up-regulate CD11c on NK cells, which was comparable to the effect of IL-15 alone (Table I). Additionally, we tested the effects of several cytokines involved in differentiation of DC (TNF-α, GM-CSF, IL-4) (27), or known to up-regulate CD11c in granulocytes (IL-8) as controls (28) in the same assay. These cytokines showed no significant effect (Table I).

$CD11c^{high}$ MS relapsed earlier

Given the significant difference in activation status and cytokine phenotype of NK cells as well as HLA-DR expression by $CD4^+$ T cells, it was particularly interesting to know whether $CD11c^{low}$ and $CD11c^{high}$ MS may follow a different clinical course. A new cohort of

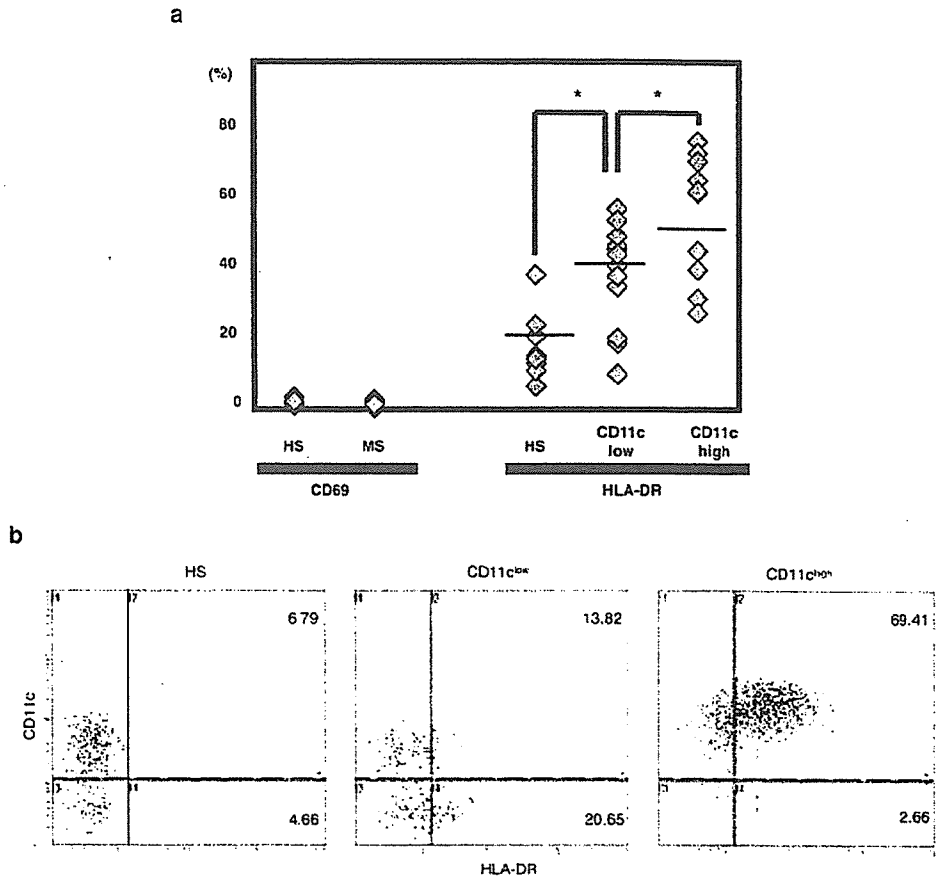

FIGURE 2. Proportions of HLA-DR$^+$ NK cells increase in CD11c^{high} MS. *a*. CD69 and HLA-DR expression on NK cells (CD3$^-$ CD56$^+$ cells). Data are expressed as proportions (percent) of CD69$^+$ cells (7 HS and 16 MS patients in remission) or HLA-DR$^+$ cells (10 HS and 25 MS patients) within whole NK cells. The Student *t* test was used for statistical analysis. Horizontal bars indicate the mean values. *, $p < 0.05$. *b*. Representative expression patterns of HLA-DR vs CD11c on NK cells from a healthy subject (*left*), CD11c^{low} MS (*middle*), and CD11c^{high} MS (*right*).

13 CD11c^{low} and 10 CD11c^{high} MS patients listed in Table II were followed for up to 120 days. In this preliminary exploration, we set the first episode of relapse after blood sampling as an end point. When the neurologist prescribed corticosteroids without knowing any information on the NK cell phenotype, the patient was considered as the dropout at that time point. Remission rate was calculated as Kaplan-Meier survival rate, and statistical difference between CD11c^{low} and CD11c^{high} MS was evaluated with the log-rank test (Fig. 5*a*). At entry, there was no significant difference in the age and disease duration between CD11c^{low} and CD11c^{high} MS (Table II). On analyzing the collected data after completing the study, we found that 8 patients developed a single relapse during the observation period and that the proportion of patients who have had relapse during the follow-up period was greatly higher in CD11c^{high} MS (6 of 10, 60%) than in CD11c^{low} MS (2 of 13, 15.3%). Furthermore, the log-rank test revealed that CD11c^{high} MS relapsed significantly earlier than CD11c^{low} MS ($p = 0.003$), suggesting a possible role of CD11c as a temporal marker for predicting relapse within months after examination. We also explored whether the difference between CD11c^{high} and CD11c^{low} could be influenced by age or sex. When we selected a group of patients younger than 38.5 years old (the mean age of all the patients), a significantly earlier relapse in CD11c^{high} than CD11c^{low} MS was confirmed in this group of patients ($p = 0.0067$, Fig. 5*b*). In the rest of the patients (<38.5 years old), the difference was less clear and not significant ($p = 0.095$). In female patients, CD11c^{high} MS relapsed significantly earlier than CD11c^{low} MS ($p = 0.035$, Fig. 5*c*), whereas this tendency was not statistically significant in male patients ($p = 0.083$). By examining the patients' medical records, we also found that the duration from the last relapse tended to be shorter in CD11c^{high} than CD11c^{low} MS (14.7 ± 12 mo in CD11c^{high} vs 26.7 ± 24.3 mo in CD11c^{low}) and that the mean number of relapses per year was higher in CD11c^{high} MS (0.9 ± 0.6 in CD11c^{high} vs 0.5 ± 0.5 in CD11c^{low}). These are consistent with the postulate that CD11c^{high} MS might be immunologically more active than CD11c^{low} MS (Table II).

Alteration of CD11c expression in the course of MS

We previously described that NK cells may lose NK2 phenotype during relapse (3). It is interesting to know whether the CD11c phenotype also changes in the course of MS. During the follow-up period of 120 days, 8 patients developed a relapse. We were able to take blood samples at relapse before treatment with corticosteroid and then compared the relapse samples with the samples obtained during remission at initiation of the study. As shown in Fig. 6, we saw an obvious tendency that the levels of CD11c expression would decline during relapse ($p < 0.05$). HLA-DR expression on NK cells was also reduced in some patients during relapse, but the difference between remission and relapse samples was not statistically significant.

Expression pattern of CD95 vs CD11c on NK cells in MS

In a previous study, we showed that MS patients could be divided into CD95^{high} and CD95^{low} according to the frequency of CD95$^+$ cells among NK cells (4). Additionally, we examined whether expression of CD11c and CD95 may independently reflect the status of MS. We found no significant correlation between CD95 (%) and CD11c (MFI) on NK cells in MS ($r = 0.29$, $p = 0.16$ with Spearman's correlation coefficient by rank test), indicating that expression of CD95 and CD11c on NK cells may be regulated independently. By setting the upper limits of CD95$^+$ (%) and CD11c MFI as (the average + 2 × SD) of HS (CD95: 44.6%, CD11c: 5.04),

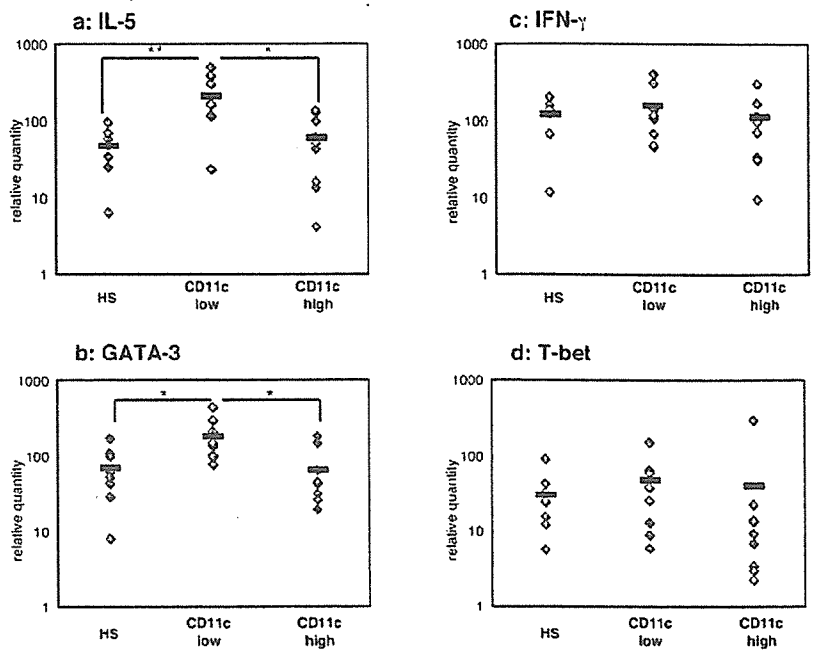

FIGURE 3. IL-5 and GATA-3 mRNA are increased in $CD11c^{low}$ but not in $CD11c^{high}$ MS. Total RNAs were extracted from purified NK cells of HS (n = 8), $CD11c^{low}$ (n = 9), or $CD11c^{high}$ MS (n = 8). mRNA expression of IL-5 (*a*), GATA-3 (*b*), IFN-γ (*c*), and T-bet (*d*) was evaluated by quantitative PCR. The data are normalized to endogenous β-actin expressions in the same samples. ANOVA was used for statistical analysis. Horizontal bars indicate the mean values. *, $p < 0.05$; **, $p < 0.01$.

we then examined whether there is a correlation between CD11c CD95 phenotype and clinical conditions (Fig. 7). Naturally, all the healthy subjects were plotted in the *left lower quadrant* ($CD95^{low}CD11c^{low}$). In contrast, MS patients were plotted in all the four quadrants with differential proportions of patients who have no relapse during 120 days: $CD95^{low}CD11c^{low}$: 3/3 (100%), $CD95^{low}CD11c^{high}$: 1/2 (50%), $CD95^{high}CD11c^{low}$: 8/10 (80%), $CD95^{high}CD11c^{high}$: 2/7 (28.6%). Although the data for $CD95^{low}$ subjects (*lower left* and *lower right*) need to be omitted due to the limited sample size, we found that the difference between $CD95^{high}CD11c^{low}$ and $CD95^{high}CD11c^{high}$ in remission rate was significant with log-rank test (p = 0.028). Provided that $CD95^{high}$ patients possessed an increased frequency of memory autoreactive T cells (4), this result is consistent with the idea that when comparable numbers of autoimmune T cells are present in the peripheral circulation, remission of MS is more stable in patients with $CD11c^{low}$ NK cells.

Discussion

Blood examination of systemic autoimmune diseases such as systemic lupus erythematosus usually exhibits measurable abnormalities such as elevation of autoantibodies, which is useful for evaluating activity of disease. In contrast, patients with MS do not accompany such systemic abnormalities in laboratory tests except

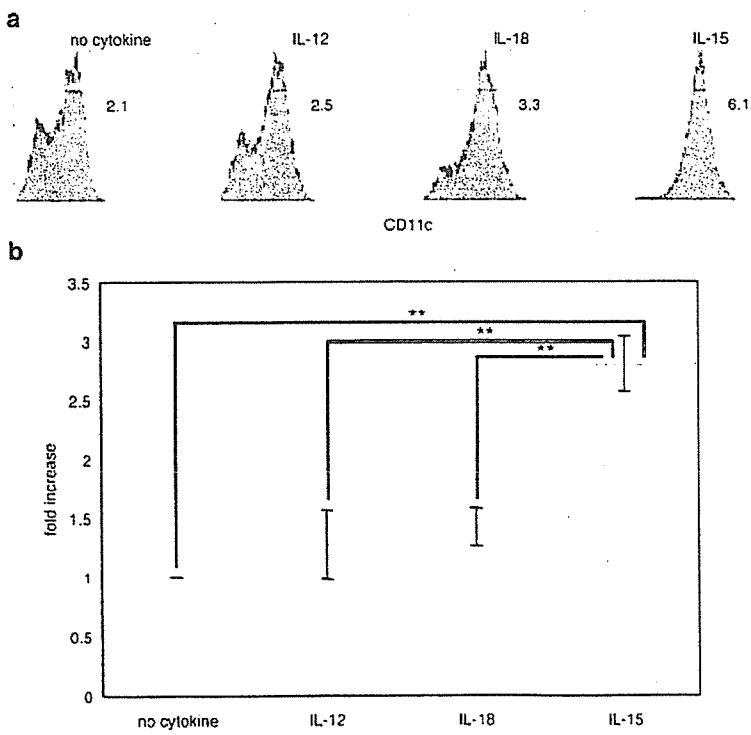

FIGURE 4. CD11c expression on NK cells is upregulated with addition of IL-15. *a*, Purified NK cells were cultured in the absence or presence of IL-12, IL-18, or IL-15. Three days later, the cells were stained with anti-CD11c-PE, -CD3-ECD, and -CD56-PC5 mAb. CD11c expression on NK cells ($CD3^-CD56^+$ cells) is demonstrated as single histogram. Values indicate CD11c MFI of $CD11c^+$ fractions. A representative of three independent experiments is shown. *b*, Data are expressed as mean fold increase of CD11c MFI (the MFI in the presence of cytokine/the MFI in the absence of cytokine) + SD from three independent experiments. ANOVA was used for statistical analysis. **, $p < 0.01$.

Table I. *Effect of several cytokines on CD11c expression on NK cells*

	No Cytokine	IL-12	IL-18	IL-15	IL-12 + IL-18	IL-4	TNF	GM-CSF	IL-23	IL-8
Expt. 1	1.00[a]	1.19	1.57	**2.90**	ND	ND	ND	ND	ND	ND
Expt. 2	1.00	1.04	1.43	**2.96**	**2.86**	ND	ND	ND	ND	ND
Expt. 3	1.00	1.59	1.25	**2.53**	**3.44**	ND	ND	ND	ND	ND
Expt. 4	1.00	ND	ND	**2.62**	ND	1.19	1.10	0.95	1.14	ND
Expt. 5	1.00	ND	ND	**2.81**	ND	1.24	ND	1.05	1.05	1.00
Mean	1.00	1.27	1.42	**2.77**	**3.15**	1.21	1.10	1.00	1.10	1.00
SD	0.00	0.29	0.16	0.19	0.41	0.03		0.07	0.07	

[a] Purified NK cells were stimulated with cytokines. Data are expressed as fold increase of CD11c MFI (the MFI in the presence of the indicated cytokines/the MFI in the absence of cytokines) in the presence of indicated cytokines. More than a 2-fold increase is highlighted (bold).

in unusual cases. It is currently recognized that autoreactive T cells might be activated and expanded to various degrees in the peripheral blood and peripheral lymphoid organs of MS even during remission (1–4). In fact, our previous work suggests that a higher number of memory autoreactive T cells is linked with unstable disease course (4). If we are able to accurately evaluate the immune status of each patient with a relatively simple test, it should be most helpful in treatment and management of MS. In this line, it is currently of particular importance to identify measurable indicators which would serve as clinically appropriate biomarkers in MS (2).

This study has clarified for the first time to our knowledge that CD11c expression on peripheral NK cells is significantly up-regulated in a major proportion of patients with MS in remission. To obtain insights into the mechanism and the biological meaning of the NK cell expression of CD11c in autoimmune disease MS, we have attempted to clarify the difference between $CD11c^{high}$ and $CD11c^{low}$ patients regarding phenotypes of NK cells, cytokine profile, and temporal clinical activity. We also explored which inflammatory cytokines might induce CD11c on NK cells. According to the NK cell expression of CD11c, we have classified the patients with MS in remission into $CD11c^{high}$ and $CD11c^{low}$. Most notably, NK2 phenotype characterized by predominant IL-5 production was seen in $CD11c^{low}$ patients, but not in $CD11c^{high}$. Consistently, the $CD11c^{high}$ patients were found to be clinically more active than $CD11c^{low}$ as judged by the remission rate during the 120 days after examination. These results indicate that up-regulation of CD11c on NK cells would reflect the temporal disease activity and therefore could be used to identify patients who are likely to exacerbate within months. It has been reported that $CD11c^{+}$ NK cells in mice could serve as APCs (6, 7). However, we could not reveal Ag presenting capacity of human $CD11c^{+}$ NK cells (data not shown).

Regarding the mechanism of CD11c induction on NK cells, we have found that in $CD11c^{high}$ patients, HLA-DR is concomitantly up-regulated with CD11c on NK cells (Fig. 2), which suggests that up-regulation of CD11c may represent an activation-induced change. After exploring the culture condition that may induce CD11c on NK cells, we have found that the addition of IL-15 or combination of IL-12 and IL-18 would increase the expression levels of CD11c on NK cells from healthy individuals. Because increased levels of these proinflammatory cytokines are detected in the blood samples of MS (11–13, 18, 19, 23), it is possible that in

Table II. *Information on the patients whose clinical courses were followed for up to 120 days*

Identification No.	Group	Age (years)	Sex	Disease Period (Years)	Total Number of Relapses	Duration from the Last Relapse (mo)	Mean Numbers of Relapse/Year
1	Low	17	F[a]	9.6	2	24	0.2
2	Low	52	M	12.2	9	3	0.7
3	Low	31	F	6.2	13	7	2.1
4	Low	32	F	3.9	1	34	0.3
5	Low	42	F	2.2	1	8	0.5
6	Low	35	M	20	3	88	0.2
7	Low	37	M	8.5	3	50	0.4
8	Low	35	F	2.4	1	38	0.4
9	Low	26	F	4.8	2	10	0.4
10	Low	26	F	1.5	1	8	0.7
11	Low	41	M	5.5	1	24	0.2
12	Low	64	F	4.5	2	8	0.4
13	Low	42	F	6.3	1	45	0.2
Mean + SD		36.9 + 12.0		6.7 + 5.0	3.1 + 3.7	26.7 + 24.3	0.5 + 0.5
14	High	39	M	4.4	2	22	0.5
15	High	31	F	9.2	11	14	1.2
16	High	46	F	7.4	>20[b]	2	ND
17	High	53	F	2.1	4	5	1.9
18	High	59	F	4.9	2	19	0.4
19	High	27	M	9.3	4	9	0.4
20	High	36	F	2.7	1	19	0.4
21	High	34	F	3.8	2	43	0.5
22	High	60	F	3.4	6	10	1.8
23	High	21	F	1.8	2	4	1.1
		40.6 + 13.4		4.9 + 2.8	3.8 + 3.1	14.7 + 12.0	0.9 + 0.6

[a] F, Female; M, male.
[b] This value is eliminated from calculation of the mean.

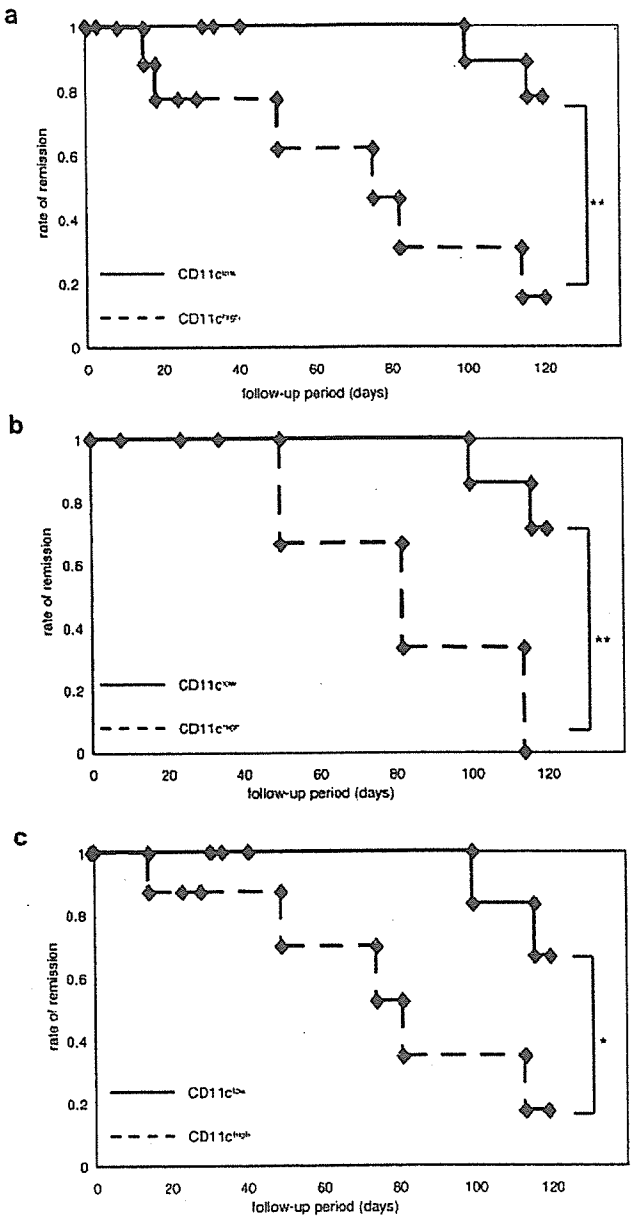

FIGURE 5. Rate of remission is lower in $CD11c^{high}$ MS. The first episode of relapse after blood sampling was set as an end point and clinical course of each patient was followed for up to 120 days. The remission rate was calculated in all (*a*), the younger (*b*), or female (*c*) patients as Kaplan-Meier survival rate, and statistical difference between $CD11c^{low}$ and $CD11c^{high}$ MS was evaluated with log-rank test at day 120. *, $p < 0.05$; **, $p < 0.01$.

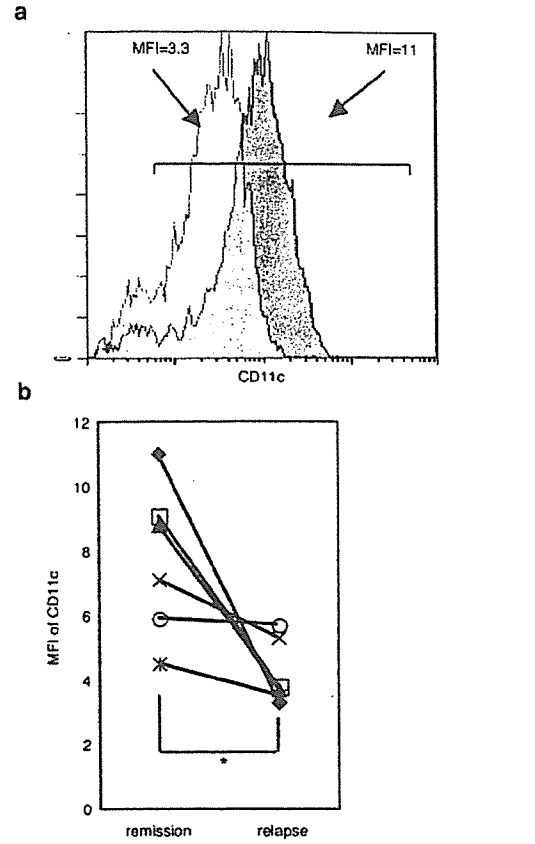

FIGURE 6. Down-regulation of CD11c expression during relapse. *a*, Representative CD11c histograms from the same patient in remission (closed) and relapse (open). Values indicate CD11c MFI of $CD11c^+$ fractions. *b*, Comparison of NK cells from remission and relapse from the same patients ($n = 6$). The data obtained from the same patients are connected with lines. Wilcoxon signed-ranks test was used for statistical analysis. *, $p < 0.05$.

vitro CD11c induction on NK cells may recapitulate the phenotypic alteration of NK cells in $CD11c^{high}$ patients. Interestingly, IL-18 is not only a cytokine able to facilitate IFN-γ production by NK cells in cooperation with IL-12 (25, 26) but is crucial in inducing pathogenic autoimmune responses (21). Furthermore, autoimmune encephalitogenic T cells can induce more serious disease upon adoptive transfer when they are preactivated in the presence of IL-12 and IL-18 (20). Taken together, these results allow us to speculate that the proinflammatory cytokines may be involved in the up-regulation of CD11c on NK cells. Although the relationship between serum cytokine concentration and levels of CD11c expression on NK cells should be estimated in future studies, a previous work (11, 29, 30) showing that a probable link between IL-15 and temporal disease activity, indicates that NK cell expression of CD11c is likely to correlate with the levels of cytokines.

In the Th cell differentiation, specific transcription factors have been identified that play a crucial role in inducing Th1 or Th2 cells. Namely, Th1 differentiation characterized by IFN-γ induction requires a transcription factor T-bet, whereas GATA-3 and c-*maf* act to promote Th2 cytokine production (31–33). Human NK cells cultured in the presence of IL-12 or IL-4 differentiate into NK1 or NK2 populations, reminiscent of Th1 and Th2 cells (5). Whereas NK1 cells produce IL-10 and IFN-γ, NK2 cells would serve as immune regulators by producing IL-5 and IL-13. Notably, up-regulation of GATA-3 has been reported in mouse NK2 cells (17), raising a possibility that Th cells and NK cells might share the same transcription factor for inducing the key cytokine. We have previously reported that IL-5 expression is one of the characteristics of NK cells in the remission state of MS (3). However, it was not excluded that overexpression of IL-5 could be restricted to a proportion of the patients. Here, we have addressed whether NK cells from $CD11c^{high}$ and $CD11c^{low}$ may differ with regard to expression levels of IFN-γ and IL-5 and of their transcription factors T-bet and GATA-3. By measuring the mRNAs, we found that expression levels of IL-5 and GATA-3 are elevated in $CD11c^{low}$ MS but not in $CD11c^{high}$ (Fig. 3). Furthermore, we showed that

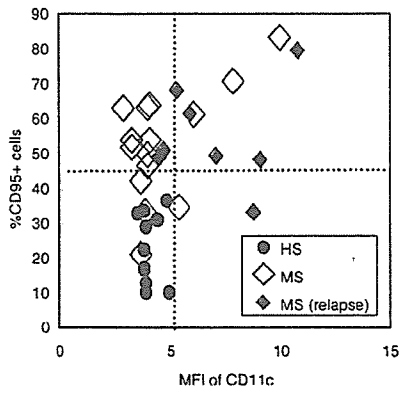

FIGURE 7. Expression pattern of CD95 vs CD11c on NK cells from MS. PBMC from MS or HS were stained with CD95-FITC, CD11c-PE, CD3-ECD, and CD56-PC5. After determining the proportion of $CD95^+$ cells among NK cells and CD11c expression (MFI) of CD11c, we plotted each patient according to the obtained values. Dotted lines represent the upper limits of $CD95^+$ cell (percent) and CD11c MFI for HS as (the average + two times SD) of HS. ●, HS; ◇, MS; ◆, MS patients who relapsed during the 120 days follow-up period.

neither IFN-γ nor T-bet was increased in $CD11c^{high}$ MS. This suggests that NK cells from $CD11c^{low}$ are NK2-biased but those from $CD11c^{high}$ are not, although MS in remission as a whole is NK2-biased as compared with control subjects. More recently, we have observed that stimulation with IL-15 or IL-12 plus IL-18 would decrease IL-5 and GATA-3 mRNA in purified NK cells with reciprocal up-regulation of CD11c (data not shown). This further supports a model that proinflammatory cytokines may play a crucial role in the absence of NK2 bias in $CD11c^{high}$ MS.

To clarify the clinical differences between $CD11c^{high}$ and $CD11c^{low}$, we followed up the clinical course of the patients after blood sampling. Although there was no significant difference in clinical parameters at examination of NK cells, we have found that $CD11c^{high}$ MS showed a significantly earlier relapse than $CD11c^{low}$ MS. This is consistent with our assumption that the absence of NK2 bias in $CD11c^{high}$ MS should imply that regulatory NK cell functions are defective in this group of patients. When we reanalyzed the data regarding various clinical parameters, we found that an earlier relapse in $CD11c^{high}$ than $CD11c^{low}$ MS is more remarkable in the younger group (<38.5 years old) or in female patients. Furthermore, the duration from the last relapse tended to be shorter and the mean number of relapses per year higher in $CD11c^{high}$ MS, supporting that $CD11c^{high}$ MS is more active than $CD11c^{low}$ MS.

When we analyzed expression of CD95 and CD11c on NK cells simultaneously, we found that MS patients in remission could be divided into four subgroups (Fig. 7). When we compared clinical course after examination of NK cell phenotypes, we found that $CD95^{high}CD11c^{high}$ MS relapsed significantly earlier than $CD95^{high}CD11c^{low}$ MS ($p = 0.028$ with log-rank test). This result indicates that $CD95^{high}CD11c^{high}$ MS may be most unstable subgroup of MS, among the patients whose clinical state could be judged as being in clinical remission.

In this study, we have demonstrated that MS patients differentially express CD11c on peripheral blood NK cells and a higher expression of CD11c on NK cells may reflect the temporal disease activity as well as functional alteration of regulatory NK cells. Our results have a clinical implication because of a lack of appropriate biomarker to monitor the immunological status in MS at present. To verify the reliability of this marker, longitudinal examination of CD11c expression on NK cells in the same patients should be performed in the future study.

Disclosures

The authors have no financial conflict of interest.

References

1. Sospedra, M., and R. Martin. 2005. Immunology of multiple sclerosis. *Annu. Rev. Immunol.* 23: 683–747.
2. Bielekova, B., and R. Martin. 2004. Development of biomarkers in multiple sclerosis. *Brain* 127: 1463–1478.
3. Takahashi, K., S. Miyake, T. Kondo, K. Terao, M. Hatakenaka, S. Hashimoto, and T. Yamamura. 2001. Natural killer type 2 bias in remission of multiple sclerosis. *J. Clin. Invest.* 107: R23–R29.
4. Takahashi, K., T. Aranami, M. Endoh, S. Miyake, and T. Yamamura. 2004. The regulatory role of natural killer cells in multiple sclerosis. *Brain* 127: 1917–1927.
5. Peritt, D., S. Robertson, G. Gri, L. Showe, M. Aste-Amezaga, and G. Trinchieri. 1998. Differentiation of human NK cells into NK1 and NK2 subsets. *J. Immunol.* 161: 5821–5824.
6. Homann, D., A. Jahreis, T. Wolfe, A. Hughes, B. Coon, M. J. van Stipdonk, K. R. Prilliman, S. P. Schoenberger, and M. G. von Herrath. 2002. CD40L blockade prevents autoimmune diabetes by induction of bitypic NK/DC regulatory cells. *Immunity* 16: 403–415.
7. Pillarisetty, V. G., S. C. Katz, J. I. Bleier, A. B. Shah, and R. P. Dematteo. 2005. Natural killer dendritic cells have both antigen presenting and lytic function and in response to CpG produce IFN-γ via autocrine IL-12. *J. Immunol.* 174: 2612–2618.
8. Bilsland, C. A., M. S. Diamond, and T. A. Springer. 1994. The leukocyte integrin p150,95 (CD11c/CD18) as a receptor for iC3b: activation by a heterologous β subunit and localization of a ligand recognition site to the I domain. *J. Immunol.* 152: 4582–4589.
9. Stacker, S. A., and T. A. Springer. 1991. Leukocyte integrin P150,95 (CD11c/CD18) functions as an adhesion molecule binding to a counter-receptor on stimulated endothelium. *J. Immunol.* 146: 648–655.
10. Morelli, A. E., A. T. Larregina, W. J. Shufesky, A. F. Zahorchak, A. J. Logar, G. D. Papworth, Z. Wang, S. C. Watkins, L. D. Falo, Jr., and A. W. Thomson. 2003. Internalization of circulating apoptotic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. *Blood* 101: 611–620.
11. Blanco-Jerez, C., J. F. Plaza, J. Masjuan, L. M. Orensanz, and J. C. Alvarez-Cermeno. 2002. Increased levels of IL-15 mRNA in relapsing-remitting multiple sclerosis attacks. *J. Neuroimmunol.* 128: 90–94.
12. Huang, W. X., P. Huang, and J. Hillert. 2004. Increased expression of caspase-1 and interleukin-18 in peripheral blood mononuclear cells in patients with multiple sclerosis. *Mult. Scler.* 10: 482–487.
13. Balashov, K. E., D. R. Smith, S. J. Khoury, D. A. Hafler, and H. L. Weiner. 1997. Increased interleukin 12 production in progressive multiple sclerosis: induction by activated $CD4^+$ T cells via CD40 ligand. *Proc. Natl. Acad. Sci. USA* 94: 599–603.
14. Karni, A., D. N. Koldzic, P. Bharanidharan, S. J. Khoury, and H. L. Weiner. 2002. IL-18 is linked to raised IFN-γ in multiple sclerosis and is induced by activated $CD4^+$ T cells via CD40-CD40 ligand interactions. *J. Neuroimmunol.* 125: 134–140.
15. McDonald, W. I., A. Compston, G. Edan, D. Goodkin, H. P. Hartung, F. D. Lublin, H. F. McFarland, D. W. Paty, C. H. Polman, S. C. Reingold, et al. 2001. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann. Neurol.* 50: 121–127.
16. Lima, M., J. Almeida, M. dos Anjos Teixeira, M. L. Queiros, B. Justica, and A. Orfao. 2002. The "ex vivo" patterns of CD2/CD7, CD57/CD11c, CD38/CD11b, CD45RA/CD45RO, and CD11a/HLA-DR expression identify acute/early and chronic/late NK-cell activation states. *Blood Cells Mol. Dis.* 28: 181–190.
17. Katsumoto, T., M. Kimura, M. Yamashita, H. Hosokawa, K. Hashimoto, A. Hasegawa, M. Omori, T. Miyamoto, M. Taniguchi, and T. Nakayama. 2004. STAT6-dependent differentiation and production of IL-5 and IL-13 in murine NK2 cells. *J. Immunol.* 173: 4967–4975.
18. Nicoletti, F., R. Di Marco, K. Mangano, F. Patti, E. Reggio, A. Nicoletti, K. Bendtzen, and A. Reggio. 2001. Increased serum levels of interleukin-18 in patients with multiple sclerosis. *Neurology* 57: 342–344.
19. Fassbender, K., A. Ragoschke, S. Rossol, A. Schwartz, O. Mielke, A. Paulig, and M. Hennerici. 1998. Increased release of interleukin-12p40 in MS: association with intracerebral inflammation. *Neurology* 51: 753–758.
20. Ito, A., A. Matejuk, C. Hopke, H. Drought, J. Dwyer, A. Zamora, S. Subramanian, A. A. Vandenbark, and H. Offner. 2003. Transfer of severe experimental autoimmune encephalomyelitis by IL-12- and IL-18-potentiated T cells is estrogen sensitive. *J. Immunol.* 170: 4802–4809.
21. Shi, F. D., K. Takeda, S. Akira, N. Sarvetnick, and H. G. Ljunggren. 2000. IL-18 directs autoreactive T cells and promotes autodestruction in the central nervous system via induction of IFN-γ by NK cells. *J. Immunol.* 165: 3099–3104.
22. Takeda, K., H. Tsutsui, T. Yoshimoto, O. Adachi, N. Yoshida, T. Kishimoto, H. Okamura, K. Nakanishi, and S. Akira. 1998. Defective NK cell activity and Th1 response in IL-18-deficient mice. *Immunity* 8: 383–390.

23. Makhlouf, K., H. L. Weiner, and S. J. Khoury. 2001. Increased percentage of IL-12$^+$ monocytes in the blood correlates with the presence of active MRI lesions in MS. *J. Neuroimmunol.* 119: 145–149.
24. Waldmann, T. A. 2004. Targeting the interleukin-15/interleukin-15 receptor system in inflammatory autoimmune diseases. *Arthritis Res. Ther.* 6: 174–177.
25. Fehniger, T. A., M. H. Shah, M. J. Turner, J. B. VanDeusen, S. P. Whitman, M. A. Cooper, K. Suzuki, M. Wechser, F. Goodsaid, and M. A. Caligiuri. 1999. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J. Immunol.* 162: 4511–4520.
26. Okamura, H., S. Kashiwamura, H. Tsutsui, T. Yoshimoto, and K. Nakanishi. 1998. Regulation of interferon-γ production by IL-12 and IL-18. *Curr. Opin. Immunol.* 10: 259–264.
27. Young, J. W., P. Szabolcs, and M. A. Moore. 1995. Identification of dendritic cell colony-forming units among normal human CD34$^+$ bone marrow progenitors that are expanded by c-*kit*-ligand and yield pure dendritic cell colonies in the presence of granulocyte/macrophage colony-stimulating factor and tumor necrosis factor α. *J. Exp. Med.* 182: 1111–1119.
28. Detmers, P. A., S. K. Lo, E. Olsen-Egbert, A. Walz, M. Baggiolini, and Z. A. Cohn. 1990. Neutrophil-activating protein 1/interleukin 8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. *J. Exp. Med.* 171: 1155–1162.
29. Kivisakk, P., D. Matusevicius, B. He, M. Soderstrom, S. Fredrikson, and H. Link. 1998. IL-15 mRNA expression is up-regulated in blood and cerebrospinal fluid mononuclear cells in multiple sclerosis (MS). *Clin. Exp. Immunol.* 111: 193–197.
30. Pashenkov, M., M. Mustafa, P. Kivisakk, and H. Link. 1999. Levels of interleukin-15-expressing blood mononuclear cells are elevated in multiple sclerosis. *Scand. J. Immunol.* 50: 302–308.
31. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell.* 100: 655–669.
32. Szabo, S. J., B. M. Sullivan, C. Stemmann, A. R. Satoskar, B. P. Sleckman, and L. H. Glimcher. 2002. Distinct effects of T-bet in TH1 lineage commitment and IFN-γ production in CD4 and CD8 T cells. *Science* 295: 338–342.
33. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89: 587–596.

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Biochemical and Biophysical Research Communications 340 (2006) 263–267
BBRC
www.elsevier.com/locate/ybbrc

In vivo delivery of small interfering RNA targeting brain capillary endothelial cells

Taro Hino [a,1], Takanori Yokota [a,*], Shingo Ito [b], Kazutaka Nishina [a], Young-Sook Kang [b,c], Shinobu Mori [b], Satoko Hori [b], Takashi Kanda [a], Tetsuya Terasaki [b], Hidehiro Mizusawa [a,1]

[a] *Department of Neurology and Neurological Science, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan*
[b] *Department of Molecular Biopharmacy and Genetics, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan*
[c] *College of Pharmacy, Sookmyung Women's University, Seoul, Republic of Korea*

Received 24 November 2005
Available online 9 December 2005

Abstract

Brain capillary endothelial cells (BCECs) play an important role in blood–brain barrier (BBB) functions and pathophysiologic mechanisms in brain ischemia and inflammation. We try to suppress gene expression in BCECs by intravenous application of small interfering RNA (siRNA). After injection of large dose siRNA with hydrodynamic technique to mouse, suppression of endogenous protein and the BBB function of BCECs was investigated. The brain-to-blood transport function of organic anion transporter 3 (OAT3) that expressed in BCECs was evaluated by Brain Efflux Index method in mouse. The siRNA could be delivered to BCECs and efficiently inhibited endogenously expressed protein of BCECs. The suppression effect of siRNA to OAT3 is enough to reduce the brain-to-blood transport of OAT3 substrate, benzylpenicillin at BBB. The in vivo siRNA-silencing method with hydrodynamic technique may be useful for the study of BBB function and gene therapy targeting BCECs.

Keywords: Small interfering RNA; Blood–brain barrier; Organic anion transporter 3; Brain ischemia; Brain inflammation; Drug delivery system

In brain ischemia and inflammation, the brain capillary endothelial cells (BCECs) have no longer been regarded as an inert vascular lining that is injured and morphologically changed, but actively play many important roles of these pathophysiologic mechanisms. The inhibition of signaling molecule in BCECs of vascular endothelial growth factor (VEGF)-induced vasogenic edema can reduce an ischemic lesion [1]. The inflammatory cell adhesion molecules expressed in BCECs induced by ischemia, such as intercellular adhesion molecule (ICAM) and E-selectin, can be a target molecule [2,3] for the therapy of these diseases. Because leukocytes activation and adhesion to BCECs are believed to contribute to additional, secondary neuronal injury after reperfusion [4] and initiate immune-mediated encephalopathy such as multiple sclerosis [5]. Endothelial nitric oxide synthases expressed in BCECs are also a possible target molecule. In cerebral ischemia, nitric oxide is increased and works as a prooxidant via peroxynitrite [6]. Therefore, BCECs are an important platform in the cerebral ischemia and inflammation, and express many constitutively or transiently expressed molecules which might be a therapeutic target for these pathologies.

RNA interference is a powerful tool for post-transcriptional gene silencing. Recently, we showed an in vitro model whose function of the transporter protein expressed in BCECs is inhibited by siRNA [7]. Here, we try to introduce siRNA by hydrodynamic, intravenous injection method from mouse tail vein and investigate the siRNA effect on brain-to-blood transport function by inhibiting organic anion transporter 3 (OAT3) with Brain Efflux Index method.

* Corresponding author. Fax: +81 3 5803 0169.
E-mail address: tak-yokota.nuro@tmd.ac.jp (T. Yokota).
[1] 21st century COE Program on Brain Integration and it's Disorders.

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.11.173

Materials and methods

Effect of siRNA on expression of recombinant OAT3 in culture cells. The mOAT3cDNA was subcloned from pGEM-HEN/Roct (OAT3) [8] into the *Renilla* luciferase expression vector, psiCHECK-1 (Promega).

Human embryonic kidney 293 (HEK293) cells were transfected with 80 ng of *Renilla* luciferase-fused OAT3 expression vector, 20 ng of *firefly* luciferase expression vector (pGL3; Promega), and 25 nM siRNA in each well of 24-well plates. *Renilla* luciferase activity was normalized with *firefly* luciferase activity. The luciferase activities were analyzed after 24 h after transfection using the Dual Luciferase System (Promega).

Effect of siRNA on uptake of OAT3 substrate in culture cells. The mOAT3 cDNA was subcloned into the pcDNA3 vector. HEK293 cells in 6-well plates were transfected by 0.5 μg of pcDNA3/OAT3 or vector alone with 100 nM siRNA using the Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the cells were passaged into the 24-well plates, and after another 24 h the cells were washed with phosphate-buffered saline (PBS). The uptake study was initiated at 37 °C by applying 200 μl PBS containing 0.5 μCi [^{3}H]benzylpenicillin to estimate the volume of adherent water. After incubation for 2 min, the radioactivities of ^{3}H in the cells were measured. The uptake of [^{3}H]benzylpenicillin was expressed as the ratio to control siRNA (shuffle sequence).

Animals. Adult male of Institute of Cancer Research (ICR) mice, weighing 35–42 g and age 9–10 weeks, were purchased from Charles River Laboratories. All experiments were approved by the Animal Experiment Committee of Tokyo Medical and Dental University.

In vivo transduction of siRNA with hydrodynamic injection method. Hydrodynamic injection method has been performed according to a previously reported method in mice [9]. The 50 μg siRNA in a volume equivalent to 5–10% of the body weight was rapidly injected in 3–5 s into the mouse tail vein. For comparison, the same amount of siRNA in 0.2 ml PBS was injected slowly in more than 60 s into the mouse tail vein as a regular intravenous injection method.

Brain small vascular fractionation and Western blot analysis. Mice brains were harvested 24 h after application of 50 μg siRNA SOD1 with the hydrodynamic or regular injection method. The total brain homogenate [10] and the brain vascular fraction of small vessels were prepared using a modified method reported previously [11]. Briefly, brains were homogenized in Dulbecco's modified Eagle's medium (DMEM). The homogenates were dissociated further with 0.005% (wt/vol) dispase (grade 1; Roche Diagnostic) at 37 °C for 2 h. After centrifugation (800*g*, 5 min), the pellets were suspended with a dextran solution (M_w 70,000; 15% wt/vol; Sigma) and centrifuged (4 °C, 4500*g* for 10 min). The pellets were resuspended with 0.05 M PBS for 10 min. After centrifugation (800*g*, 5 min), the final pellets of small vessels were resuspended in lysis buffer (20 mM Tris–HCl, 0.1% SDS, and 1% Triton).

Fractionated mouse brain tissues and mouse brain capillary endothelial cell line [12] cells were homogenized in buffer containing 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 4% Chaps, 1 mM phenylmethylsulfonyl fluoride (PMSF), and a protease-inhibitor cocktail (Complete-Mini; Roche Diagnostic). The 2.5 μg samples were separated with 7.5% SDS–polyacrylamide mini-gel (Bio-Rad) and transferred to a polyvinylidene difluoride membrane. The membrane was probed with anti-glucose-transporter-1 antibodies (Alpha Diagnostic International) or anti-SOD1 antibodies (Stressgen Biotechnologies) and visualized by using an ECL Western blot system (Amersham–Pharmacia).

Assay for efflux function of OAT3 in vivo. Fifty micrograms of siRNA OAT3 or control siRNA was delivered to brain capillary endothelial cells with hydrodynamic injection via the tail vein. After 36 h, the in vivo brain efflux experiments were carried out using Brain Efflux Index (BEI) method as described previously [13]. Each mouse was anesthetized intramuscularly with a mixture of ketamine (125 mg/kg) and xylazine (1.22 mg/kg), then mounted on a stereotaxic frame (SRS-6; Narishige), to hold the head in position. Using a dental drill, a bore hole was made 3.8 mm lateral to the bregma. Then, extracellular fluid buffer (122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 10 mM D-glucose, and 10 mM Hepes, pH 7.4) containing 96 nCi [^{3}H]benzylpenicillin and 4.8 nCi [^{14}C]inulin was injected over a period 1 min using a 5.0-μl microsyringe (Hamilton Reno) fitted with a fine needle at a depth of 2.5 mm from the surface of the scalp, i.e., the secondary somatosensory cortex 2 (S2) region. The needle was left in this configuration for an additional 4 min to prevent reflux of the injected solution along the injection track, before being slowly retracted. After 40 min, the whole brain was subsequently removed and the left cerebrum was isolated. After weighing each of these, tissue samples were solubilized in 2 N NaOH at 60 °C for 1 h and then mixed with Hionic-fluor (Packard). The radioactivity in each sample was assayed in a liquid scintillation counter equipped with an appropriate crossover correction for ^{3}H and ^{14}C (LS-6500; Beckman).

The BEI was defined by Eq. (1) and the percentage of substrate remaining in the ipsilateral cerebrum was determined from Eq. (2).

$$\mathrm{BEI}(\%) = \frac{\text{test substrate undergoing efflux at the BBB}}{\text{test substrate injected into the brain}} \times 100 \quad (1)$$

$$\text{100-BEI}(\%) = \frac{(\text{amount of test substrate in the brain}/\text{amount of reference in the brain})}{(\text{concentration of test substrate injected}/\text{concentration of reference injected})} \times 100. \quad (2)$$

The percentage of [^{3}H]benzylpenicillin remaining in the brain is given by (100-BEI).

The data were used when the remaining amount of [^{14}C]inulin in the brain was more than 15% of the injected amount. No significant difference was observed in the remaining percentage of [^{14}C]inulin, which is a non-permeable marker, among all samples (#1, 39.7 ± 3.5%; #2, 27.5 ± 3.4%; #3, 31.4 ± 2.9%; #2 shuffle, 28.9 ± 2.2%) (ANOVA), showing that the hydrodynamic injection of siRNA did not damage the integrity of BBB.

Data analysis. All data represent means ± SEM. An unpaired, two-tailed Student's *t* test was used to determine the significance of differences between two group means. (The difference is certified when $P < 0.05$.)

Results

siRNA directed against the OAT3 and SOD1 genes

Sense sequences of the siRNA designed to OAT3 and SOD1 genes are described as follows. The siRNA of shuffle sequence of siRNA OAT3 #2 and siRNA against unrelated gene, GBV-B virus, were used as negative controls. Uppercase letters at 3′ end indicate deoxyribonucleotides.

siRNA OAT3 #1:	5′-ucuacaacagcaccagagaTT-3′
siRNA OAT3 #2:	5′-ccauuaucuugaauguggaTT-3′
siRNA OAT3 #3:	5′-aaacaaagcaggagccagaTT-3′
siRNA-shuffle sequence:	5′-agugguaaugucuauauccTT-3′
siRNA-unrelated control:	5′-agugguaaugucuauauccTT-3′
siRNA SOD1:	5′-gguggaaaugaagaaaguaTT-3′

Effect of siRNA on expression and function of recombinant OAT3 in culture cells

siRNA OAT3 #2 most effectively reduced the expression of OAT3 in HEK293 cells by 86.2% on luciferase activity compared with control siRNA with shuffle sequence of siRNA OAT3 #2 (Fig. 1). siRNA OAT3 #1 and #3 were moderately effective.

To investigate the inhibition effect of siRNA OAT3 to its efflux function in vitro, we measured uptake of OAT3 substrate, [^{3}H]benzylpenicillin. After expression of OAT3

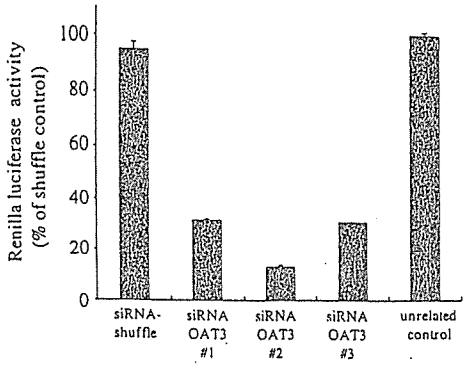

Fig. 1. Effect of siRNAs directed against the OAT3 in vitro. HEK293 cells were transfected with *Renilla* luciferase-fused OAT3 expression vector, *firefly* luciferase expression vector, and 25 nM siRNA. Reduction effect of *Renilla* luciferase activity relative to *firefly* luciferase activity was analyzed. Negative controls were the siRNA with randomized sequence of siRNA OAT3 #2 (siRNA-shuffle) and the siRNA against unrelated gene. Data were averaged from three experiments with SEM indicated.

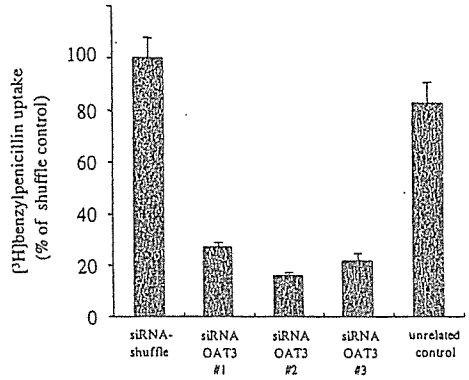

Fig. 2. Effect of siRNAs on uptake of OAT3 substrate in culture cells. Effect of siRNAs OAT3 on the OAT-3-mediated [^{3}H]benzylpenicillin uptake in HEK293 cells. After expression of OAT3 to the cells, [^{3}H]benzylpenicillin uptake was performed at 2 min, reflecting the initial uptake phase. All siRNAs were used at a concentration of 100 nM. Each value represents the mean ± SEM ($n = 4$). The increased uptake by expression of OAT3 was significantly reduced by siRNA OAT3 compared to siRNA-shuffle and siRNA-unrelated control. ($p < 0.0001$).

to HEK293 cells, the uptake mediated OAT3 was increased, and siRNA OAT3 #2 significantly inhibited the increased uptake of the substrate in HEK293 cells, compared with siRNA-shuffle and siRNA-unrelated control (Fig. 2).

In vivo delivery of siRNA to brain endothelial cells

We biochemically investigated an inhibitory effect of siRNA on expression of endogenous protein in BCECs using brain vascular fraction of small vessels from mouse brain.

For detection of endogenous protein in BCECs, we used SOD1 and siRNA to SOD1, because we have confirmed the efficient in vivo effect of this siRNA to endogenous mouse SOD1 in the siRNA-overexpressed transgenic mouse [14].

Western blot of the mouse brain small vascular fraction showed a reduction of endogenous mouse SOD1 level after hydrodynamic injection of siRNA SOD1 (Fig. 3A, left), whereas SOD1 level in the total homogenate of brain did not change (data not shown). There was a potentially more significant level of reduction on a per-BCEC basis, because the brain small vascular fraction contained proteins from cells other than BCECs such as pericytes and astrocytes [15]. We roughly estimated the content of BCECs in the brain small vascular fraction by performing a Western blot analysis with antibody to glucose-transporter-1 (GLUT-1) which specifically expressed in BCECs (Fig. 3B). The band intensity of GLUT-1 in the brain small vascular fraction was 4.1 (± 0.58) times more than that in mouse brain capillary endothelial cell lines which we previously established [12] (Fig. 3B). Since the cell line contains more than 1/8 of GLUT-1 [12], around 50% protein of the brain small vascular fraction that we made was supposed to come from brain endothelial cells.

In contrast, there was not obvious reduction of SOD1 level in the small vascular fraction after a regular intrave-

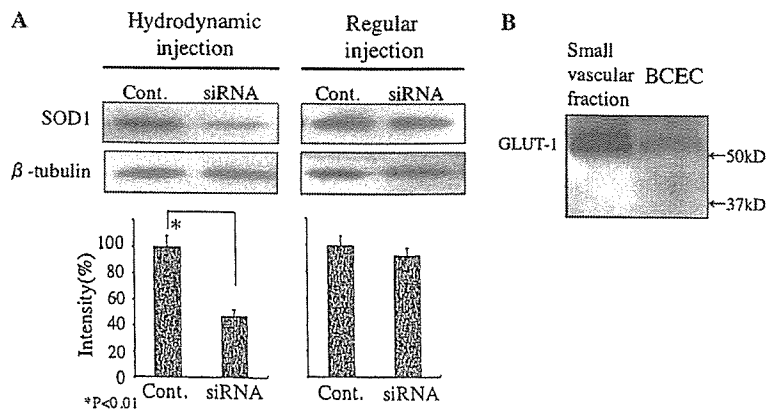

Fig. 3. Western blot analysis of mouse brain capillary-rich fraction. (A) The mouse brain small vascular fraction was examined on Western blot analysis after hydrodynamic (left) and regular (right) injection of 50 μg siRNA SOD1. The lower panels indicate percentages of signal intensities of SOD1 normalized with that of tubulin. (B) Western blot analysis with 2.5 μg protein of anti-GLUT-1 antibody of the mouse brain small vascular fraction (left) and mouse brain capillary endothelial cell lines (right). Signal intensity of GLUT-1 in the mouse brain small vascular fraction is 4.1 (± 0.58) times more than that in mouse brain capillary endothelial cell lines. BCEC, brain capillary endothelial cell line cells.

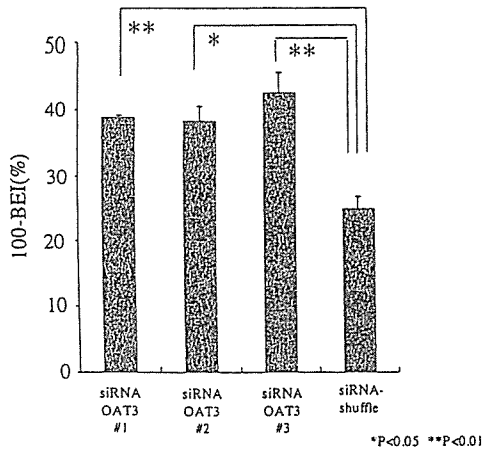

Fig. 4. Effect of siRNA on transport function of OAT3 by BEI. The 50 μg siRNA dissolved in the 5–10% volume PBS of mouse body weight was rapidly injected into the tail vein 36 h before the BEI assay. The residual radioactivity of OAT3 substrate, [^{3}H]benzylpenicillin in the brain, was measured at 40 min after intracerebral injection.

nous injection (Fig. 3A, right). These results indicate that hydrodynamic injection method is effective for delivery of siRNA to brain capillary endothelial cells.

In vivo effect of siRNA on transporter function of OAT3 in vivo

The in vivo inhibitory effect of siRNA OAT3 on the brain-to-blood efflux transport was examined with BEI method with intracerebral injection of OAT3 substrate, [^{3}H]benzylpenicillin. We intravenously injected siRNA OAT3 #2 to 11 mice, siRNA OAT3 #1 and #3 to 3 mice each, and siRNA-shuffle (control) to 7 mice with hydrodynamic method. Transport function of OAT3 was evaluated by BEI method at 36 h after the injection of siRNA. The results of 100-BEI, percentage of OAT3 substrate remaining in the brain after injection, are shown in Fig. 4. The value of 100-BEI of siRNA OAT3 #2 is significantly higher than that of siRNA-shuffle by 26.4%. Those of siRNA OAT3 #1 and #3 were also similarly higher than that of control. The results that plural different siRNAs to the OAT3 gene similarly increased 100-BEI value indicated that these siRNA influences were not "off-target effect" on molecules other than OAT3 in the brain. Taken together, these results suggested that in vivo applied-siRNA to OAT3 could suppress the brain-to-blood efflux function of OAT3.

Discussion

This is the first report of successful in vivo inhibition of endogenous gene in BCECs by systemic intravenous injection of siRNA. Furthermore, we demonstrated that our gene silencing effect was enough to suppress the transport function of OAT3 endogenously expressed in BCECs at BBB. We could deliver siRNA to BCECs by hydrodynamic injection method, but not by regular intravenous injection from the mouse tail vein. It has been thought that a rapid injection of a large bolus of solution develops a high pressure in the inferior vena cava, causing retrograde movement of the solution to the abdominal organs including liver and kidneys. Such a sharp increase in venous pressure enlarges the liver fenestrae and promotes membrane permeability of the hepatocytes, making siRNA enter the cells [16]. Since BCECs are circulated from the tail vein via lung capillary, the phasic hydrodynamic pressure in the inferior vena cava should decrease in the lung. However, rapid loading of extremely large volume of solution, 40–80% of circulating plasma volume should considerably increase hydrostatic pressure in the carotid artery due to volume overload. In addition, the rapid injection of large volume solution prevents the solution from being mixed with the serum containing RNase and keeps the concentration of siRNA extremely high when it is delivered to BCECs.

This in vivo knockdown method with siRNA to BCECs is expected to be a powerful tool for investigating function of BBB. The BBB is formed by the tight intercellular junctions of BCECs and regulates CNS homeostasis and drug delivery by restricting the transfer of substances between the circulating blood and the brain [17]. We have developed Brain Efflux Index as a reliable in vivo method of analyzing efflux transport at the BBB [18]. The efflux function of a transporter protein expressed in BCECs, such as OAT3, can be well evaluated by combining in vivo knockdown method with siRNA and BEI method.

Since synthetic siRNA does not work in the cells for no more than six days [19], long-term silencing of the target gene is necessary for investigating other functions of BCECs in the pathophysiology of atherosclerosis and Alzheimer's disease. Long-standing gene suppression can be achieved in vivo with adenovirus and adeno-associated virus (AAV) vectors expressing short hairpin RNA (shRNA) [20,21]. Actually, with the adenovirus expressing shRNA to SOD1 gene (2.0×10^9 pfu), we could efficiently suppress the endogenous SOD1 level of brain capillary-rich fraction by regular intravenous injection into mouse tail vein (unpublished data). For the evaluation of BCEC function, however, the AAV may be better than adenovirus, because BBB function should be less affected due to limited local immune response to the AAV capsid [22].

The hydrodynamic injection does not cause marked injury to organs in the animals [23], but it is hard to be clinically applied to patients because of its extremely high hydrostatic pressure and volume overload. Possible alternate is a regional delivery of large dose siRNA into carotid artery, but development of less invasive systemic delivery system in vivo is necessary for a therapeutic application of siRNA. Novel cationic liposomes have been reported to transduce efficiently siRNA into the liver [24] as well as tumor tissue [25]. These siRNAs formulated with cationic liposomes also induce interferons and cytokines in vivo through toll-like receptors [26,27] which should change the BBB function. Recently, the lipid-conjugated siRNA

at the 5′-end of the sense strand enhanced cellular uptake and gene silencing [28]. Combined with chemical modification of 2′-O-methylation and phosphorothioate to stabilize siRNA, substantial gene silencing in the liver and jejunum was achieved by a regular intravenous injection into the mouse tail vein [29]. Now, we are trying to use these new siRNA delivery methods to achieve more effective, stable, and safe gene suppression in BCECs for a clinical application.

Acknowledgments

We thank Dr. Tadashi Kanouchi and Miss Tsubura Takahashi for their help. This work was supported by grants from the Ministry of Education, Science and Culture of Japan and from the Ministry of Health, Labor and Welfare of Japan.

References

[1] R. Paul, Z.G. Zhang, B.P. Eliceiri, Q. Jiang, A.D. Boccia, R.L. Zhang, M. Chopp, D.A. Cheresh, Src deficiency or blockade of Src activity in mice provides cerebral protection following stroke, Nat. Med. 7 (2001) 222–227.

[2] C.J. Frijns, L.J. Kappell, Inflammatory cell adhesion molecules in ischemic cerebrovascular disease, Stroke 33 (2002) 2115–2122.

[3] J.J. Archelos, S. Jung, M. Mäurer, M. Schmied, H. Lassmann, T. Tamatani, M. Miyasaka, K.V. Toyka, H.P. Hartung, Inhibition of experimental autoimmune encephalomyelitis by an antibody to the intercellular adhesion molecule ICAM-1, Ann. Neurol. 34 (1993) 145–154.

[4] G.J. del Zoppo, T. Mabuchi, Cerebral microvessel responses to focal ischemia, J. Cereb. Blood Flow Metab. 23 (2003) 879–894.

[5] D.H. Miller, O.A. Khan, W.A. Sheremata, L.D. Blumhardt, G.P. Rice, M.A. Libonati, A.J. Willmer-Hulme, C.M. Dalton, K.A. Miszkiel, P.W. O'Connor, International Natalizumab Multiple Sclerosis Trial Group, A controlled trial of natalizumab for relapsing multiple sclerosis, N. Engl. J. Med. 348 (2003) 15–23.

[6] P.H. Chan, Reactive oxygen radicals in signaling and damage in the ischemic brain, J. Cereb. Blood Flow Metab. 21 (2001) 2–14.

[7] S. Hori, S. Ohtsuki, M. Ichinowatari, T. Yokota, T. Kanda, T. Terasaki, Selective gene silencing of rat ATP-binding cassette G2 transporter in an in vitro blood-brain barrier model by short interfering RNA, J. Neurochem. 93 (2005) 63–71.

[8] S. Ohtsuki, T. Kikkawa, S. Mori, S. Hori, H. Takanaga, M. Otagiri, T. Terasaki, Mouse reduced in osteosclerosis transporter function as an organic anion transporter 3 and is localized at albuminal membrane of blood-brain barrier, J. Pharmacol. Exp. Ther. 309 (2004) 1273–1281.

[9] F. Liu, Y.K. Song, D. Liu, Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA, Gene Ther. 6 (1999) 1258–1266.

[10] T. Yokota, K. Igarashi, T. Uchihara, K. Jishage, H. Tomita, A. Inaba, Y. Li, M. Arita, H. Suzuki, H. Mizusawa, H. Arai, Delayed-onset ataxia in mice lacking alpha-tocopherol transfer protein: model for neuronal degeneration caused by chronic oxidative stress, Proc. Natl. Acad. Sci. USA 98 (2001) 15185–15190.

[11] T. Kanda, H. Yoshino, T. Ariga, M. Yamawaki, R.K. Yu, Glycosphingolipid antigens in cultured microvascular bovine brain endothelial cells: sulfoglucuronyl paragloboside as a target of monoclonal IgM in demyelinative neuropathy, J. Cell Biol. 126 (1994) 235–246.

[12] K. Hosoya, K. Tetsuka, K. Nagase, M. Tomi, S. Saeki, S. Ohtsuki, T. Terasaki, Conditionally immortalized brain capillary endothelial cell lines established from a transgenic mouse harboring temperature-sensitive Simian Virus 40 large T-antigen gene, AAPS Pharmsci. 2 (2000), [article 27].

[13] A. Kakee, T. Terasaki, Y. Sugiyama, Brain efflux index as a novel method of analyzing efflux transport at the blood-brain barrier, J. Pharmacol. Exp. Ther. 277 (1996) 1550–1559.

[14] Y. Saito, T. Yokota, T. Mitani, K. Ito, M. Anzai, M. Miyagishi, K. Taira, H. Mizusawa, Trangenic small interfering RNA halts amyotrophic lateral sclerosis in a mouse model, J. Biol. Chem. 2005 (in press).

[15] T. Kanda, T. Ariga, H. Kubodera, H.L. Jin, K. Owada, T. Kasama, M. Yamawaki, H. Mizusawa, Glycosphingolipid composition of primary cultured human brain microvascular endothelial cells, J. Neurosci. Res. 78 (2004) 141–150.

[16] G. Zhang, X. Gao, Y.K. Song, R. Vollmer, D.B. Stolz, J.Z. Gasiorowski, D.A. Dean, D. Liu, Hydroporation as the mechanism of hydrodynamic delivery, Gene Ther. 11 (2004) 675–682.

[17] K. Hosoya, S. Ohtsuki, T. Terasaki, Recent advances in the brain-to-blood efflux transport across the blood-brain barrier, Int. J. Pharm. 248 (2002) 15–29.

[18] T. Terasaki, S. Ohtsuki, S. Hori, H. Takanaga, E. Nakashima, K. Hosoya, New approaches to in vitro models of blood-brain barrier drug transport, Drug Discov. Today 8 (2003) 944–954.

[19] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (2001) 494–498.

[20] H. Xia, Q. Mao, H.L. Paulson, B.L. Davidson, siRNA-mediated gene silencing in vitro and in vivo, Nat. Biotechnol. 20 (2002) 1006–1010.

[21] H. Xia, Q. Mao, S.L. Eliason, S.Q. Harper, I.H. Martins, H.T. Orr, H.L. Paulson, L. Yang, R.M. Kotin, B.L. Davidson, RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia, Nat. Med. 10 (2004) 816–820.

[22] M.G. Kaplitt, P. Leone, R.J. Samulski, X. Xiao, D.W. Pfaff, K.L. O'Malley, M.J. During, Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain, Nat. Genet. 8 (1994) 148–154.

[23] J.E. Knapp, D. Liu, Hydrodynamic delivery of DNA, Methods Mol. Biol. 245 (2004) 245–250.

[24] D.R. Sorensen, M. Leirdal, M. Sioud, Gene silencing by systemic delivery of synthetic siRNAs in adult mice, J. Mol. Biol. 327 (2003) 761–766.

[25] J. Yano, K. Hirabayashi, S. Nakagawa, T. Yamaguchi, M. Nogawa, I. Kashimori, H. Naito, H. Kitagawa, K. Ishiyama, T. Ohgi, T. Irimura, Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer, Clin. Cancer Res. 10 (2004) 7721–7726.

[26] A.D. Judge, V. Sood, J.R. Shaw, D. Fang, K. McClintock, I. MacLachlan, Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA, Nat. Biotechnol. 23 (2005) 457–462.

[27] V. Hornung, M. Guenthner-Biller, C. Bourquin, A. Ablasser, M. Schlee, S. Uematsu, A. Noronha, M. Manoharan, S. Akira, A. de Fougerolles, S. Endres, G. Hartmann, Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7, Nat. Med. 11 (2005) 263–270.

[28] C. Lorenz, P. Hadwiger, M. John, H.P. Vornlocher, C. Unverzagt, Steroid and lipid conjugates of siRNAs to enhance cellular uptake and gene silencing in liver cells, Bioorg. Med. Chem. Lett. 14 (2004) 4975–4977.

[29] J. Soutschek, A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R.K. Pandey, T. Racie, K.G. Rajeev, I. Rohl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan, H.P. Vornlocher, Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs, Nature 432 (2004) 173–178.

Journal of Clinical Immunology, Vol. 26, No. 6, November 2006 (© 2006)
DOI: 10.1007/s10875-006-9043-1

Type I Interferons Attenuate T Cell Activating Functions of Human Mast Cells by Decreasing TNF-α Production and OX40 Ligand Expression While Increasing IL-10 Production

TOMOKO FUJITA,[1] NAOTOMO KAMBE,[2] TAKASHI UCHIYAMA,[1] and TOSHIYUKI HORI[1,3]

Received May 20, 2006; accepted August 22, 2006
Published online: 19 September 2006

Recent studies have demonstrated that mast cells not only mediate inflammatory reactions in type I allergy but also play an important role in adaptive immunity. In the present study, we investigated the effects of interferon-α, which shares the same receptor as IFN-β, on human cord blood-derived mast cells. Mast cells produced TNF-α, and IL-10, and expressed OX40 ligand upon activation by crosslinking of FcεRI. When treated with interferon-α, TNF-α production was decreased while IL-10 and TGF-β productions were increased. Furthermore, flow cytometric analysis revealed that interferon-α downregulated expression OX40 ligand on mast cells which is crucial for mast cell-T cell interaction. We confirmed that the viability of mast cells was not affected by interferon-α treatment. Accordingly, interferon-α-treated mast cells induced lower levels of $CD4^+$ T cell proliferation compared with those without interferon-α treatment. These results suggest that type I interferons suppress T cell immune responses through their regulatory effects on mast cells.

KEY WORDS: Mast cells; $CD4^+$ T cells; Type I interferons; OX40 ligand; .

INTRODUCTION

Mast cells (MCs) are well known for their ability to mediate inflammatory reactions in pathological processes of allergic diseases (1). Upon recognition of specific antigens by IgE bound to FcεRI, MCs secrete various kinds of cytokines, chemokines, and chemical mediators, which trigger allergic reactions (2). This IgE-dependent activation of MCs has been emphasized as a major causative event of harmful type I hypersensitivity. However, MCs are originally engaged in the first line defense against bacterial as well as parasite infections. Recent evidence has indicated that in mice, MCs recruit and activate T cells in the draining lymph nodes during bacterial infection and thus positively regulate adaptive immunity (3). TNF-α has been shown to play a pivotal role in this enforcement of T cell responses (3, 4). In addition to TNF-α secretion, MCs can directly interact with T cells and even present antigens to T cells in the context of MHC class I and class II (5–7), resulting in enhanced T cell proliferation. It has been reported that human MCs and murine BMMCs express OX40 ligand (OX40L) upon activation and the OX40/OX40L system plays a crucial role in MCs-mediated T cell proliferation (8, 9).

In clinical settings, MCs are implicated in the pathogenesis of most allergic diseases in which Th2-type immune responses appear to be predominant such as bronchial asthma and atopic dermatitis (10). On the other hand, recent reports have revealed that MCs also play important roles in some Th1 dominant diseases such as inflammatory bowel diseases (11), multiple sclerosis (MS) (12), and experimental autoimmune encephalomyelitis (EAE), an animal model of MS. MS is a demyelinating disorder of the central nervous system that preferentially affects young adults. Although myelin-specific $CD4^+$ T cells are principal effector cells, other cell types are also likely to be involved in the pathogenesis of the disease. It is noted that MCs are required for the development and aggravation of EAE because MC-deficient W/W^v mice are less sensitive to EAE than wild-type mice and reconstitution of MCs by injection of bone marrow-derived MCs from wild-type mice restore the severity of EAE(13). In humans, MCs are found in the CNS plaques of MS patients (14) and

[1]Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, Kyoto, Japan.
[2]Department of Dermatology, Graduate School of Medicine, Kyoto University, 54 Shogoin-Kawaracho, Sakyoku, Kyoto 606-8507, Japan.
[3]To whom correspondence should be addressed to Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, 54 Shogoin-Kawaracho, Sakyoku, Kyoto 606-8507, Japan; e-mail: thori@kuhp.kyoto-u.ac.jp.

the concentrations of histamine and tryptase were higher in MS patients compared with normal healthy individuals (15, 16), suggesting that MCs are also involved in MS.

Treatment of MS remains to be a challenging problem. Apart from several immunosuppressive drugs, type I IFNs have been shown to be effective in a proportion of MS patients (17), although the precise mechanism of their effects has not been fully elucidated. In a study using specimens from MS patients, IFN-β has been reported to correct Th1/Th2 imbalance by increasing IL-4 production and lowering IFN-γ production (18). Since type I IFNs do not have such a direct effect on $CD4^+$ T cells, these reports suggest the presence of other target cells of type I IFNs that mediate alleviation of pathological T cell responses. Based on the roles of MCs in EAE and MS as discussed above, it is to be determined whether type I IFNs could modify MC-mediated T cell activation processes. As far as we know, effects of IFNs on MCs have not been studied in detail. In the present study, we addressed this question by using human cord blood-derived MCs and IFN-α, a type I IFN. Herewith we report that IFN-α did not affect the viability of MCs but decreased TNF-α production and OX40 ligand expression while increased TGF-β and IL-10 production. Thus, type I IFNs indirectly attenuate T cell responses through the regulatory effects on MCs. The possible involvement of MCs in the effectiveness of IFN-β therapy for MS and other Th1 diseases is discussed.

MATERIALS AND METHODS

Antibodies and Cytokines

SCF and IL-6 were purchased from Wako (Tokyo, Japan) and IL-3 and IL-4 were from Peprotech (Rock Hill, NJ). Human myeloma IgE was from Chemicon International (Temecula, CA) and goat anti-human IgE from Biosource (Camarillo, CA). Phycoerythrin (PE)-conjugated anti-human OX40 ligand monoclonal antibody (mAb) (clone ik-1) and PE-conjugated mouse IgG1 was obtained from Becton-Dickinson (Franklin Lakes, NJ). Anti-OX40L blocking mAb (mouse IgG2a, clone ik-5) was generated in our laboratory (19). Control IgG2a (clone UPC10) was purchased from Sigma (St. Louis, MO).

Preparation of MCs

Human cord blood-derived MCs (CBMCs) were generated as described (20). In brief, mononuclear cells were isolated from cord blood of healthy neonates with the informed consent of the parents and subsequently cultured in AIM-V (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Life Technologies, Rockville, MD), 100 U/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml L-glutamine (penicillin–streptomycin–glutamine; Life Technologies), 100 ng/ml SCF and 50 ng/ml IL-6. Half medium change was performed once a week. Cells cultured for more than 8 weeks were used which consisted of >95% pure MCs measured as c-kit-positive cells by flow cytometry.

Activation of MCs

Naïve MCs were cultured with 5 ng/ml IL-3 and 10 ng/ml IL-4 for 5 days. Human IgE was added at 1 μg/ml for the last 24 h to be presensitized. For the aggregation of FcεRI, presensitized MCs were collected, washed three times, and cultured with 2 μg/ml goat anti-human IgE Ab in SCF-containing medium (without IL-6) in the presence or absence of IFN-α for the indicated periods of time.

RT-PCR

Presensitized MCs were cultured with anti-IgE Ab and with or without IFN-α (Biosource, Camarillo, CA) for 6 h. Cells were then collected and washed three times. Total RNA was isolated with RNeasy mini kit (QIAGEN, Hilden, Germany). 1 μg of RNA was used in reverse transcription using ImProm-II™ reverse transcription system (Promega, Madison, WI). The same amount of complementary DNA was used in the subsequent PCR. The following primers were used: β-actin (sense 5′-TCAGCCTCTTCTCCTTCCTGATCG-3′; antisense 5′-TGAAGAGGACCTGGGAGTAGATGA-3′) (21), TNF-α (5′-GTTCCTCAGCCTCTTCTCCT-3′; antisense 5′-ATCTATCTGGGAGGGGTCT T-3′) (22), IL-10 (sense 5′-ATGCCCCAAGCTGAGAAC CAAGACC CA-3′; antisense 5′-AAGGGGCTGGGTCAGCTATCCCA-3′) (23), and TGF-β (sense 5′-CTACTAGGCCAAGGA GGTCAC-3′; antisense 5′-TTGCTGAGGTATGGCCAG GAA-3′) (24). PCR products were separated through 1.5% agarose gel, stained with ethidium bromide, and visualized with an UV transilluminator.

Measurement of Cytokine Production

Cytokine production was measured by ELISA according to the manufacture's protocol. Presensitzed MCs were treated with goat anti-human IgE Ab and IFN-α for 6 h at 1.0×10^6/ml. Culture supernatants were then collected and stored at −80°C until they were subjected to analysis. Concentrations of TNF-α, IL-10, and TGF-β in the supernatants were measured by using the respective ELISA kits (Biosource).

MTT Assay

2×10^4 presensitized CBMCs were activated with goat anti-IgE Ab in the presence or absence of IFN-α in a total volume of 100 μl/well in a 96 well flat-bottomed plate (IWAKI, Tokyo, Japan). 10 μl cell count reagent SF (Nakalai, Kyoto, Japan) per well was added after 24 h and MCs were further incubated for 1 h. Optical density values at 450 nm (OD 450) were measured with a microplate reader (BioRad, Hercules, CA).

Flow Cytometric Analysis of OX40L Expression

MCs were presensitized and activated in the presence or absence of 100 ng/ml IFN-α. After 24 h, cells were collected and stained with either PE-conjugated anti-human OX40L mAb or PE-conjugated mouse IgG_1 (Becton-Dickinson). After immunofluorescence staining, cells were analyzed with a FACScan flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences).

$CD4^+$ T Cell Proliferation Assay

To avoid the influence of IFN-α on $CD4^+$ T cells, MCs that had been presensitized and treated with anti-IgE Ab and IFN-α were washed thoroughly and suspended in RPMI1640 supplemented with 10% FBS. Since MCs are unable to proliferate in the absence of SCF, we used MCs without any anti-proliferative treatment such as irradiation and exposure to mitomycin C that might affect the functions of MCs. $CD4^+$ T cells were purified with CD4 isolation kit (Miltenyi Biotech) from PBMC of normal healthy donors. The purity of $CD4^+$ T cells was >95% in all assays performed. In a 96-well round-bottomed plate (IWAKI, Tokyo, Japan), 1×10^5 $CD4^+$ T cells were cultured with 1×10^5 anti-CD3-coated beads (Dynabeads CD3, Dynal Biotech, Oslo, Norway), and activated MCs (at MC/T ratios of 1/5, 1/10, and 1/20) in a total volume of 200 μl. To evaluate the involvement of the OX40/OX40L system, some cultures were set up in the presence of either 50 μg/ml ik-5 or control IgG2a. Cells were cultured in triplicate for 5 days and pulsed with [^{3}H]thymidine ([^{3}H]TdR) (0.5 μCi/well; MEN Life Science, Boston, MA) for the last 6 h of culture. After harvesting cells, the incorporated radioactivity was measured in a liquid scintillation counter (Packard Instrument Company, Downers Grove, IL).

Statistical Analysis

Statistical analyses were performed by Student's t-test or paired t-test. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

TNF-α Production was Decreased While IL-10 Production was Increased in IFN-α-Treated MCs

MCs are known to release various kinds of cytokines and chemokines upon activation. Among these, TNF-α is crucial in MC-$CD4^+$ T interaction (4). IL-10 is an immunoregulatory cytokines which inhibit T cell proliferation. Therefore, we focused on these cytokines and investigated their production in IFN-α-treated MCs. Expression of TNF-α mRNA was decreased while IL-10 and TGF-β mRNA were increased in IFN-α-treated MCs (Fig. 1a). To confirm these results, we measured the concentrations of TNF-α, IL-10 and TGF-β in the supernatants of activated MCs preactivated with or without IFN-α by ELISA. As shown in Fig. 1b, pretreatment with IFN-α decreased the production of TNF-α in a dose-dependent manner. Three independent experiments were done with different CBMC batches to give similar results. The decrease in TNF-α production by IFN-α (at 100 ng/ml) was statistically significant. Conversely, pretreatment with IFN-α increased IL-10 production reproducibly (Fig. 1c). This increase was also statistically significant. TGF-β production was slightly increased by IFN-α pretreatment, but the difference was not statistically significant (data not shown).

IFN-α Treatment did not Affect the Survival of MCs

It is known that the proliferation of human bone marrow-derived MCs was not affected by IFN-α treatment (25). However, the effect of IFN-α on the survival of CBMC has not been reported. To exclude the possibility that the decrease in cytokine production was due to impaired survival of CBMCs, we performed MTT assay of CBMCs cultured with various concentrations of IFN-α. As shown in Fig. 2, IFN-α pretreatment did not affect the survival of CBMCs at any concentrations tested.

Pretreatment with IFN-α Suppresses OX40L Expression on Activated MCs

OX40 is a costimulatory molecule that potently promotes $CD4^+$ T cell proliferation. Its ligand, OX40L, has been reported to be expressed on human tonsillar and PBMC-derived MCs upon crosslinking of FcεRI and play a vital role in MC-$CD4^+$ T cell interaction although the expression of OX40L on CBMCs has not been reported. In $OX40L^{-/-}$ mice, The severity of EAE was markedly attenuated, whereas it was significantly enhanced in OX40L

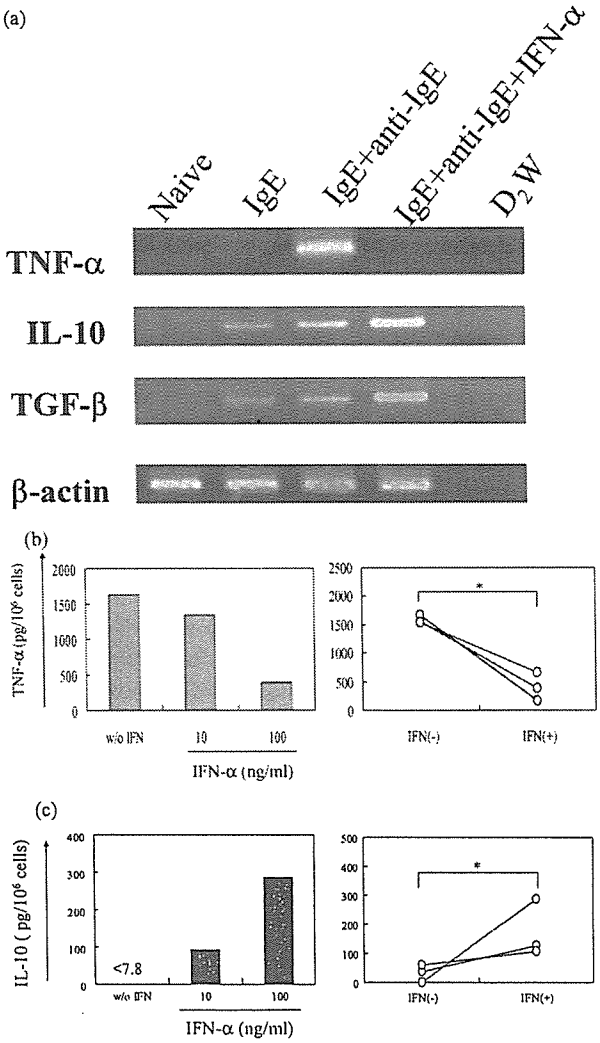

Fig. 1. (a) RT-PCR for TNF-α, IL-10, and TGF-β. PCR products were electrophoresed on 1.5% agarose gel. Data shows a representative of three independent experiments. (b) and (c) ELISA assay of TNF-α and IL-10. TNF-α and IL-10 productions of presensitized CBMCs activated in the presence or absence of IFN-α were measured by ELISA. A representative of three independent experiments for each cytokine is shown (left). The difference in each cytokine production (pg/ml) between the absence and presence of 100 ng/ml IFN-α was analyzed statistically. $^*p < 0.05$.

transgenic mice (26). If MCs participate in the pathogenesis of EAE, it is likely that OX40L expressed on MCs are involved in such process. We first confirmed that OX40L was also expressed on CBMCs after FcεRI crosslinking as reported with tonsillar and PBMC-derived MCs and found that pretreatment with IFN-α considerably suppressed its expression (Fig. 3).

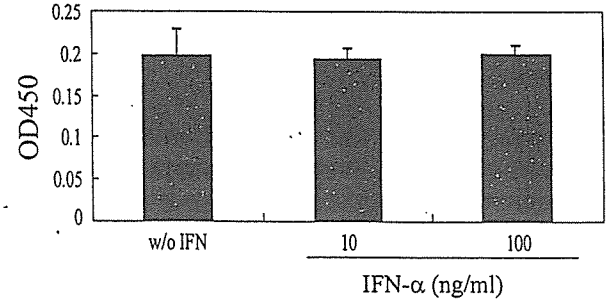

Fig. 2. Survival of IFN-α-treated MCs was measured by MTT assay. MTT assay was performed 24 h after activation with anti-IgE Ab and the indicated concentrations of IFN-α. Data indicate mean ± SD of triplicate wells of a representative of three independent experiments.

Pretreatment of MCs with IFN-α Significantly Inhibited Costimulation of CD4⁺ T Cells by CBMCsn

Finally we examined the effects of IFN-α on T cell activating functions of MCs, because TNF-α and OX40L which we showed were suppressed by IFN-α are both important factors for T cell costimulation by MCs. We cocultured $CD4^+$ T cells from normal healthy donors and preactivated MCs with or without IFN-α treatment, and analyzed T cell proliferation on day 5. In this assay, IFN-α and anti-IgE Ab were washed out before coculture in order to exclude the effect of IFN-α on $CD4^+$ T cells. As shown in Fig. 4a, IFN-α-treated MCs induced lower levels of T cell proliferation compared with non-treated MCs, indicating that IFN-α suppressed T cell activating functions of MCs. The OX40/OX40L system is reported to play a role in MC-T cell interaction in humans (8). In accordance with the previous report, an addition of anti-OX40L mAb significantly suppressed the costimulatory functions of activated CBMCs (Table I).

DISCUSSION

Accumulating evidence has indicated that MCs are not simple effector cells in allergic reactions but are multifunctional accessory cells for T cell responses that influence the magnitude and direction of adaptive immunity. Consistent with this new concept, MCs have been shown to play important roles in the pathogenesis of not only Th2 diseases but also some Th1 diseases such as inflammatory bowel disease and MS (11, 12). Thus, it is essential to understand the regulation of MC functions in order to elucidate the MC-mediated pathogenesis and control of the diseases.

In the present study, we showed that pretreatment of MCs with IFN-α decreased TNF-α production and

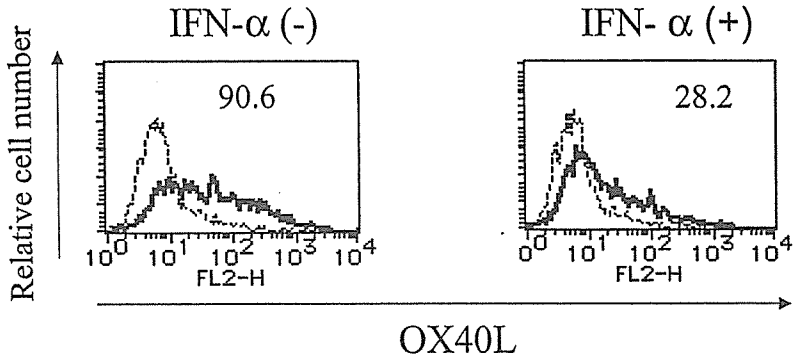

Fig. 3. OX40L expression on activated CBMCs. Presensitized MCs activated in the absence or presence of 100 ng/ml IFN-α were subjected to flow cytometric analysis. Data of a representative of three independent experiments are shown. Numbers in the histograms indicate ΔMFI of OX40L expression.

OX40L expression, while IL-10 production was increased. In accordance with these results, IFN-α significantly suppressed the costimulatory functions of MCs in coculture with $CD4^+$ T cells. In the literature, we found two primitive reports that IFN-α/β plus IFN-γ suppressed TNF-α mRNA levels in rat MC lines and rat peritoneal MCs (27), and that in vivo topical administration of IFN-α to nasal mucosa of allergic patients resulted in an decrease in TNF-α^+ mucosal MC number in the biopsy specimens (28). In these reports, however, the effects of type I IFNs on actual TNF-α secretion, on other cytokine productions, on OX40L expression, or on T cell costimulatory functions were not investigated. Thus, the effects of IFN-α on MC functions have never been studied in detail. As far as we know, the present study is the first to delineate the entire picture of the effects of IFN-α on the MC functions.

IFN-α-induced increase in IL-10 production by MCs is particularly important because IL-10 negatively affects immune responses by inhibiting the production of inflammatory cytokines. IL-10 is reported to suppress the release of inflammatory cytokine by MCs in an autocrine manner (20). Thus, it is possible that inhibition of TNF-α production presented in this study was mediated by increased IL-10 that was induced by IFN-α treatment. In allergic reactions, the role of IL-10-secreting T(R)1 cells is reported to induce tolerance (29). However, the role of IL-10 secreted by MCs in the context of adaptive immunity has never been reported. Our results suggest the possibility that immunoregulatory cytokines derived from IFN-α-treated MCs may contribute to the suppression of costimulatory function of MCs themselves and other surrounding immune cells leading to regulation of the overall inflammatory reactions.

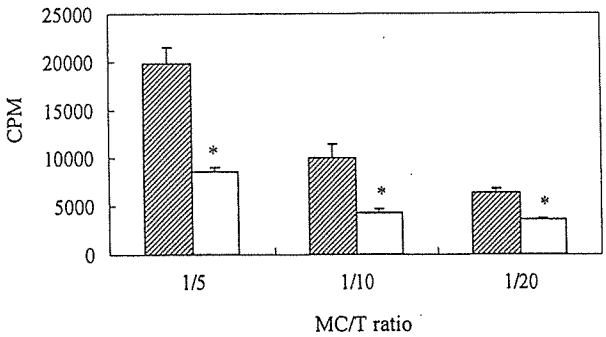

Fig. 4. Coculture of CBMCs and $CD4^+$ T from normal healthy donors. Presensitized CBMCs were activated with goat anti-IgE in the presence or absence of 100 ng/ml IFN-α for 24 h and subsequently washed. 1×10^5 T cells were cocultured with MCs at indicated MC/T ratios in the presence of anti-CD3-coated beads for 5 days. Cells were pulsed with [^{3}H]TdR for the last 6 h of culture and the incorporated radioactivity was measured by a liquid scintillation counter. Data indicate mean ± SD of triplicate wells. Striped bars indicate CBMCs activated without IFN-α and white bars indicate CBMCs activated with IFN-α. *$p < 0.05$ compared with "without IFN-α."

Table I. The Effect of Anti-OX40L mAb on the Costimulatory Activities of CBMCs

	CPM ± SD
w/o mAb	10118 ± 1303
Control	13047 ± 490
ik-5	7132 ± 350

Notes. Presensitized CBMCs were activated with IgE and anti-IgE Ab, washed, and then cocultured with $CD4^+$ T cells and anti-CD3-coated beads in the absence or presence of either 50 μg/ml ik-5 or control IgG2a for 5 days. T cell proliferation was measured by [^{3}H]TdR incorporation for the last 6 h. Three independent experiments were done and gave similar results. Data indicate the mean ± SD of triplicate cultures of a representative experiment.

Downregulation of OX40L expression on MCs by IFN-α is also impressive. OX40L can be induced on human peripheral blood stem cells-derived MCs in vitro and human MCs isolated from tonsil and lung (8). These MCs have been reported to interact directly with $CD4^+$ T cells and augment their proliferation mainly via the OX40/OX40L system. It is now recognized that expression of OX40L on antigen presenting cells that stand for mostly dendritic cells (DCs) is the key event for promotion of inflammatory responses. In fact, OX40/OX40L system is implicated in the exacerbation of EAE (26, 30). Considering that presence of MCs is required for the development of severe EAE, it is possible that type I IFNs ameliorate the manifestations of MS by suppressing the expression of OX40L on MCs.

In conclusion, we showed here that type I IFNs attenuate T cell activating functions of human MCs by decreasing TNF-α production and OX40 ligand expression while increasing IL-10 production. Effectiveness of type I IFNs in the treatment of MS is still an enigma, since IFNs are considered to be a proinflammatory cytokine enhancing Th1 immune responses. The present study provides a novel insight into the inhibitory aspects of IFNs action and presents the possibility that IFNs might influence T cell response via their regulatory effects on MCs in inflammatory Th1 diseases including MS.

ACKNOWLEDGMENTS

This work was partly supported by grants-in-aid from the Ministry of Health, Labour and Welfare of Japan.

REFERENCES

1. Galli SJ: Mast cells and basophils. Curr Opin Hematol 7:32–39, 2000
2. Kawakami T, Galli SJ: Regulation of mast-cell and basophil function and survival by IgE. Nat Rev Immunol 2:773–786, 2002
3. McLachlan JB, Hart JP, Pizzo SV, Shelburne CP, Staats HF, Gunn MD, Abraham SN: Mast cell-derived tumor necrosis factor induces hypertrophy of draining lymph nodes during infection. Nat Immunol 4:1199–1205, 2003
4. Nakae S, Suto H, Kakurai M, Sedgwick JD, Tsai M, Galli SJ: Mast cells enhance T cell activation: Importance of mast cell-derived TNF. Proc Natl Acad Sci USA 102:6467–6472, 2005
5. Frandji P, Mourad W, Tkaczyk C, Singer M, David B, Colle JH, *et al.*: IL-4 mRNA transcription is induced in mouse bone marrow-derived mast cells through an MHC class II-dependent signaling pathway. Eur J Immunol 28:844–854, 1998
6. Frandji P, Oskeritzian C, Cacaraci F, Lapeyre J, Peronet R, David B, *et al.*: Antigen-dependent stimulation by bone marrow-derived mast cells of MHC class II-restricted T cell hybridoma. J Immunol 151:6318–6328, 1993
7. Skokos D, Le Panse S, Villa I, Rousselle JC, Peronet R, David B, *et al.*: Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes. J Immunol 166:868–876, 2001
8. Kashiwakura J, Yokoi H, Saito H, Okayama Y: T cell proliferation by direct cross-talk between OX40 ligand on human mast cells and OX40 on human T cells: Comparison of gene expression profiles between human tonsillar and lung-cultured mast cells. J Immunol 173:5247–5257, 2004
9. Nakae S, Suto H, Iikura M, Kakurai M, Sedgwick JD, Tsai M, *et al.*: Mast cells enhance T cell activation: importance of mast cell costimulatory molecules and secreted TNF. J Immunol 176:2238–2248, 2006
10. Pawankar R: Mast cells in allergic airway disease and chronic rhinosinusitis. Chem Immunol Allergy 87:111–129, 2005
11. Araki Y, Andoh A, Fujiyama Y, Bamba T: Development of dextran sulphate sodium-induced experimental colitis is suppressed in genetically mast cell-deficient Ws/Ws rats. Clin Exp Immunol 119:264–269, 2000
12. Skaper SD, Facci L, Romanello S, Leon A: Mast cell activation causes delayed neurodegeneration in mixed hippocampal cultures via the nitric oxide pathway. J Neurochem 66:1157–1166, 1996
13. Secor VH, Secor WE, Gutekunst CA, Brown MA: Mast cells are essential for early onset and severe disease in a murine model of multiple sclerosis. J Exp Med 191:813–822, 2000
14. Olsson Y: Mast cells in plaques of multiple sclerosis. Acta Neurol Scand 50:611–618, 1974
15. Tuomisto L, Kilpelainen H, Riekkinen P: Histamine and histamine-*N*-methyltransferase in the CSF of patients with multiple sclerosis. Agents Actions 13:255–257, 1983
16. Roznieckí JJ, Hauser SL, Stein M, Lincoln R, Theoharides TC: Elevated mast cell tryptase in cerebrospinal fluid of multiple sclerosis patients. Ann Neurol 37:63–66, 1995
17. Virley DJ: Developing therapeutics for the treatment of multiple sclerosis. NeuroRx 2:638–649, 2005
18. Berghella AM, Totaro R, Pellegrini P, Contasta I, Russo T, Carolei A, *et al.*: Immunological study of IFNbeta-1a-treated and untreated multiple sclerosis patients: clarifying IFNbeta mechanisms and establishing specific dendritic cell immunotherapy. Neuroimmunomodulation 12:29–44, 2005
19. Imura A, Hori T, Imada K, Ishikawa T, Tanaka Y, Maeda M, *et al.*: The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells. J Exp Med 183:2185–2195, 1996
20. Royer B, Varadaradjalou S, Saas P, Gabiot AC, Kantelip B, Feger F, *et al.*: Autocrine regulation of cord blood-derived human mast cell activation by IL-10. J Allergy Clin Immunol 108:80–86, 2001
21. Carre PC, Mortenson RL, King TE, Jr., Noble PW, Sable CL, Riches DW: Increased expression of the interleukin-8 gene by alveolar macrophages in idiopathic pulmonary fibrosis. A potential mechanism for the recruitment and activation of neutrophils in lung fibrosis. J Clin Invest 88:1802–1810, 1991
22. Li DQ, Luo L, Chen Z, Kim HS, Song XJ, Pflugfelder SC: JNK and ERK MAP kinases mediate induction of IL-1beta, TNF-alpha and IL-8 following hyperosmolar stress in human limbal epithelial cells. Exp Eye Res 82:588–596, 2006
23. de Saint-Vis B, Fugier-Vivier I, Massacrier C, Gaillard C, Vanbervliet B, Ait-Yahia S, *et al.*: The cytokine profile expressed