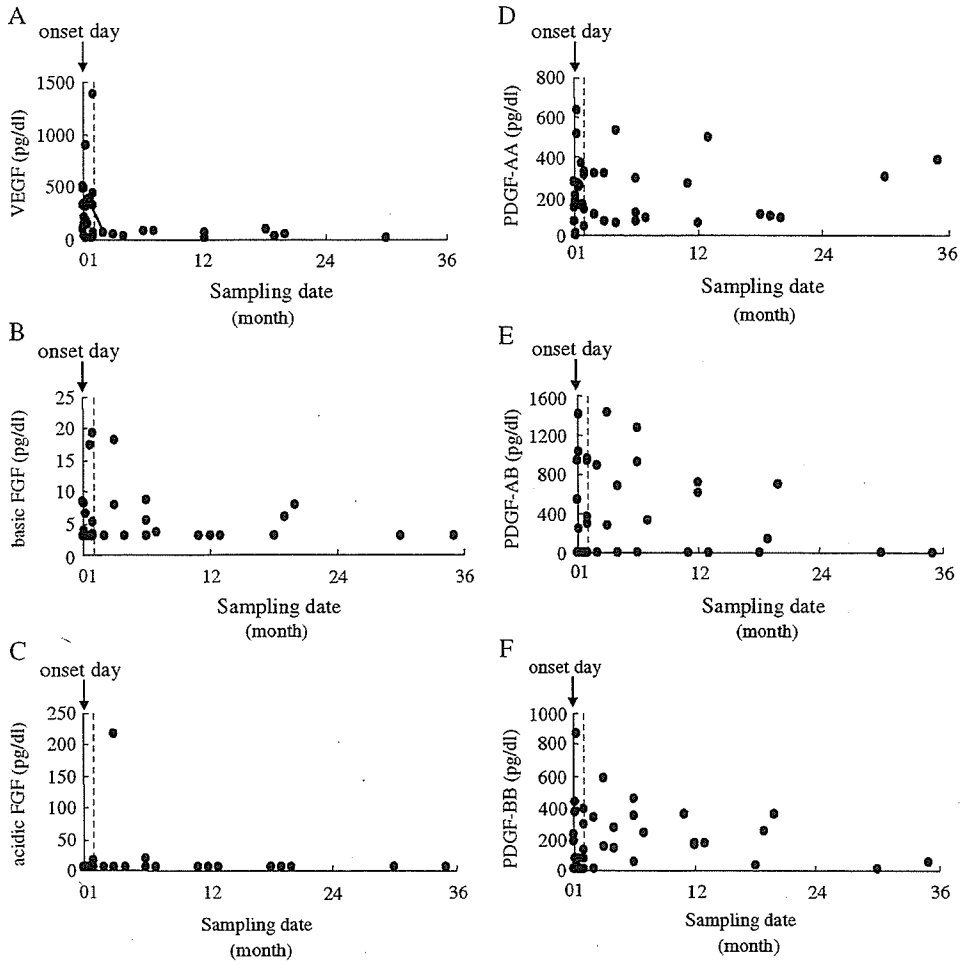

Fig. 5. Vascular growth factor levels against time intervals (months) between the onset day of relapse and blood sampling. (A) VEGF. (B) Basic FGF. (C) Acidic FGF. (D) PDGF-AA. (E) PDGF-AB. (F) PDGF-BB. Relapse phase (broken line) was determined to be within one month after the onset day (arrow). A sharp rise of VEGF levels but not of any others is shown at the time of relapse in MS.

sequentially measured at relapse and remission had elevated VEGF at relapse (333.1 pg/ml) when longitudinally extensive spinal cord lesion was present and showed a decrease in VEGF level in the following remission (67.8 pg/ml) (Figs. 1 and 5). No other vascular growth factors showed such a clear-cut elevation at relapse phase. As the two OS-MS patients with very high VEGF values were also the two oldest patients in the OS-MS group, and both had longitudinally extensive spinal cord lesions, we then analyzed the relationship of vascular growth factor levels with age and MRI findings. A significant positive correlation between VEGF level and age at examination was detected in total MS patients, OS-MS patients, and OS-MS patients in relapse and controls ($r=0.356$ and $p=0.0210$, $r=0.563$ and $p=0.0070$, $r=0.714$ and $p=0.0100$ and $r=0.397$ and $p=0.0296$, respectively), the r value being highest in OS-MS in relapse (Fig. 6). In addition, MS patients at relapse and remission had a similar tendency, yet did not reach statistical significance ($p=0.0579$ and $p=0.0688$, respectively). In OS-MS, there was also a significant positive correlation of VEGF with age at onset ($r=0.459$, $p=0.0279$), but not in C-MS. VEGF had no correlation with disease duration. VEGF at relapse had a significant positive correlation with spinal cord lesion length on MRI in total MS patients ($r=0.506$, $p=0.0319$) (Fig. 7A). However, VEGF in MS patients had no significant correlation with EDSS scores at relapse or convalescence (Fig. 7B). VEGF levels in MS patients with longitudinally extensive spinal cord lesions at relapse were significantly higher than in controls ($p=0.0411$), but not in those without such lesions (Fig. 7C). In contrast, VEGF level had no correlation with the presence of brain MRI lesions (numbers of T2-high and gadolinium-enhanced lesions). Moreover, VEGF level had no correlation with the number of black holes. Even when MS patients on low dose corticosteroids were excluded from statistical analyses, essentially the same results were obtained in respect to VEGF levels and the above-mentioned parameters (data not shown). Neither basic FGF nor any other vascular growth factors examined showed correlation with any

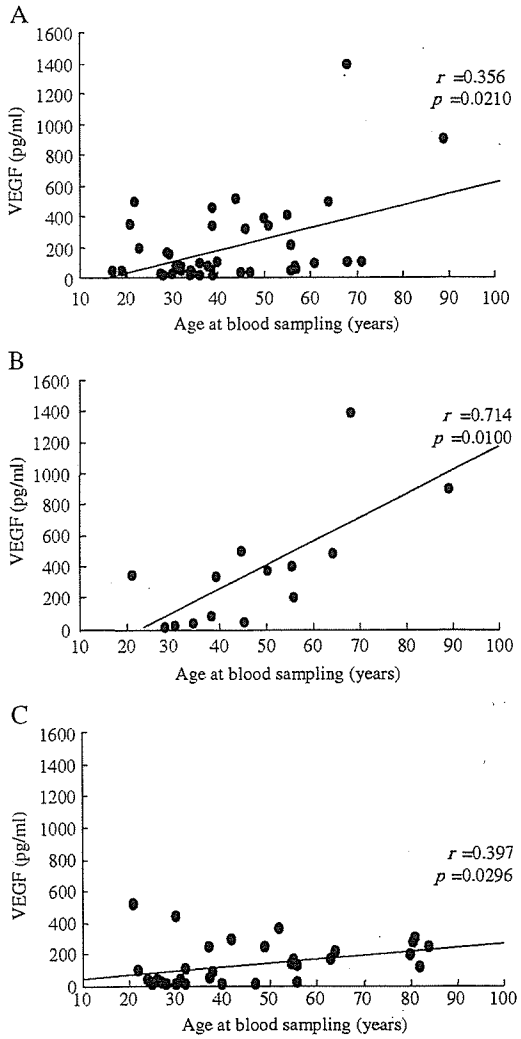

Fig. 6. (A) Relationship between VEGF concentration in sera and age at blood sampling in MS patients. (B) Relationship between VEGF concentration in sera and age at blood sampling in OS-MS patients in relapse. (C) Relationship between VEGF concentration in sera and age at blood sampling in controls.

clinical parameters, including age at onset or examination, disease duration, or MRI findings.

4. Discussion

In the present study, we found that serum VEGF concentrations were significantly elevated in MS patients in relapse compared with controls and those in remission, irrespective of clinical subtype. Furthermore, basic FGF was significantly increased in C-MS patients, irrespective of clinical phase.

In this study, only VEGF was found to be associated with MS relapse. Stockhammer et al. [15] measured serum VEGF in 19 MS patients with unspecified clinical phases and found no elevation. The difference between their results and ours may be explained in part by the sample size and probably the clinical phase of the samples, although exact reasons cannot be clarified as they did not specify the clinical phase of their samples. Proescholdt et al. [7] reported the up-regulation of VEGF in MS lesions as well as in acute lesions with experimental autoimmune encephalomyelitis, an animal model of MS. The results of that study and those in ours strongly suggest that VEGF has a critical role in MS relapse. VEGF is a major mediator of vascular permeability increases as well as angiogenesis [4], and through activation of endothelial cells has been shown to be associated with both acute and chronic inflammation, such as acute allograft rejection [16] and bronchial asthma

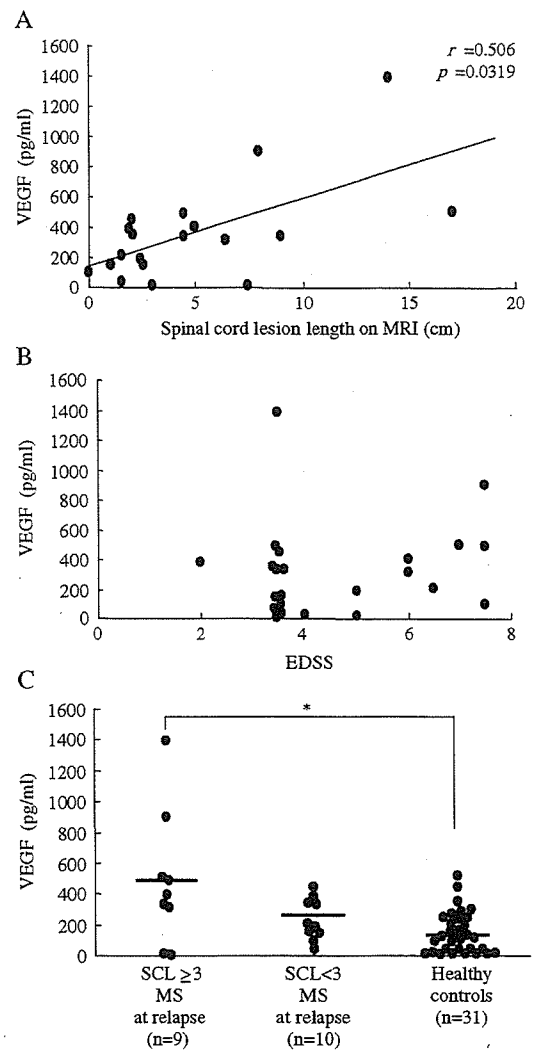

Fig. 7. (A) Relationship between VEGF concentration and spinal cord lesion length (cm) on MRI in MS patients at relapse. (B) Relationship between VEGF concentration and EDSS scores in MS patients at relapse. (C) VEGF concentration in sera from MS patients at relapse analyzed separately according to their spinal cord lesion length on MRI. Bars indicate the mean of each group. SCL≥3: spinal cord lesion length ≥3 vertebral segments, SCL<3: spinal cord lesion length <3 vertebral segments. *$p<0.05$.

[17,18], and vasculopathies, such as diabetic nephropathy [19] and retinopathy [20]. In MS lesions, endothelial cells proliferate [21] and blood vessels show irregular morphology consistent with angiogenesis [3]. Moreover, vascular permeability changes, as shown by serial gadolinium-enhanced MRI studies, precede MS lesion formation [1]. Although not all the sera examined in the present study were obtained at the very beginning of relapse, gadolinium-enhancement of MS lesions usually persists for 3 to 5 weeks [22]; therefore, enhanced VEGF responses at relapse shown here are likely to have contributed to the increased vascular permeability and subsequent endothelial cell proliferation and angiogenesis during relapse. Moreover, VEGF itself is secreted by activated T cells and induces Th1 polarization [23]. As in MS CSF cells where marked upregulation of Th1 cells is seen at relapse [24], VEGF is likely to aggravate inflammation of MS in which Th1 cells are considered to play a crucial role.

Interestingly, VEGF at relapse had a positive correlation with spinal cord lesion length on MRI but not brain MRI findings, and only MS patients with longitudinally extensive spinal cord lesions showed significantly higher VEGF levels compared with controls. These findings suggest that VEGF might contribute to the development of severe spinal cord lesions. Edema following increased vascular permeability induced by VEGF might lead to focal disturbance of microcirculation with subsequent ischemia and tissue necrosis [2]. Such a mechanism might preferentially occur in the spinal cord and optic nerves that have tight and compact structures within narrow bony canals [2].

Therefore, the effects of VEGF might be more deleterious in spinal cord than in brain, which might partly explain the cystic necrosis of tissues frequently seen in Asian MS patients. The lack of correlation between VEGF and EDSS scores might be explained by the fact that the latter are determined by dysfunction in various parts of the CNS. Since genotypes encoding higher VEGF production are reportedly associated with diabetic microvasculopathy [19,20] and acute renal allograft rejection [16], and since significant ethnic differences in polymorphisms of the VEGF gene have also been reported [25], such polymorphisms warrant future investigation in MS patients, especially Asian ones.

In our study, both MS patients in total and controls showed similar weak but significant positive correlations between VEGF and age at blood sampling, while in OS-MS in relapse VEGF and age at examination showed much stronger positive correlations. Although VEGF has been shown to be associated with age-associated diseases, such as neovascular age-related macular degeneration [4,26], no correlation between VEGF and age has previously been reported [27]. However, in these studies, such wide ranging ages as in ours have not been investigated. In general, late-onset MS is associated with faster progression of disability [28], and it has repeatedly been shown that OS-MS has a higher age at onset [10] and thus a higher age at any following relapses, along with greater disability compared with C-MS. In the present MRI study, the spinal cord lesion length also tended to correlate positively with age at examination in MS. Therefore, age-associated increases of VEGF in a general population and thus in MS patients at relapse might in part explain the severe disability and longitudinally extensive spinal cord lesions in OS-MS patients with higher ages at onset and relapse, yet its mechanism remains to be elucidated.

Among other vascular growth factors, only basic FGF was elevated in both acute and stable C-MS patients, which indicates that involvement of other vascular growth factors such as acidic FGF and PDGFs-AA, -AB, and -BB is less likely. A combined increase of VEGF and basic FGF has been reported in inflammatory bowel disease, such as Crohn's disease [28], and liver cirrhosis with spider angiomas [29]. Basic FGF could stimulate the production of VEGF and enhance angiogenesis, and its contribution to neovascularization has been postulated in the above-mentioned conditions [28,29]. However, basic FGF in the present study had no correlation with VEGF level, and no significant change in basic FGF was observed in OS-MS in which angiogenesis is prominent. Basic FGF not only contributes to angiogenesis [6], but it also induces oligodendrocyte progenitor growth in response to demyelination [5]. Therefore, since in C-MS, in which demyelination is prominent and varying degrees of remyelination occur, increases had no relationship with clinical phase and basic FGF levels might reflect host tissue repair rather than vascular changes. Clearly, further large scale studies on these growth factors in cerebrospinal fluid are necessary.

Finally, the proportion of patients who fulfilled McDonald's MRI criteria was significantly lower in OS-MS than C-MS, reflecting the low brain lesion loads that have repeatedly been described in the former [10]. On the other hand, the frequency of longitudinally extensive spinal cord lesions was significantly more common in OS-MS than C-MS. Although the increased VEGF levels at relapse in OS-MS compared with C-MS did not reach statistical significance, extremely high levels of VEGF were only observed in OS-MS patients. Therefore, it is suggested that a certain unique process resulting in distinct distribution of the CNS lesions likely occurs in OS-MS, where VEGF might play various roles.

In summary, we found an elevated VEGF response in MS at relapse, and this had a positive correlation with spinal cord lesion length. Therefore, this might underscore the importance of anti-VEGF therapies, such as those utilizing anti-VEGF monoclonal antibody [30], antisense oligonucleotide [26] and soluble VEGF receptor [31], in MS patients.

Acknowledgements

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Long-term favorable response to interferon beta-1b is linked to cytokine deviation toward the Th2 and Tc2 sides in Japanese patients with multiple sclerosis

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Abstract

To address the immune mechanism of the long-term beneficial effects of interferon beta (IFN-β), we measured the intracellular cytokine production patterns of IFN-γ, IL-4 and IL-13 in peripheral blood $CD4^+$ and $CD8^+$ T cells, which previously displayed alterations during the early course of IFN-β treatment, in 15 Japanese patients after long-term IFN-β administration. The patients were treated with IFN-β-1b 8×10^6 units given subcutaneously every other day for a mean period of 34.5±5.5 months (range: 26–43 months). During the follow-up period, 6 patients experienced 33 relapses, while the other 9 were relapse-free. The results revealed the following cytokine alterations: (1) type 2 cytokine, such as IL-4 and IL-13, were significantly increased in producing cell percentages in both $CD4^+$ ($p=0.0356$ and $p=0.0007$, respectively) and $CD8^+$ ($p=0.0231$ and $p=0.0170$, respectively) T cells while IFN-γ, a representative type 1 cytokine, was significantly decreased in the absolute producing cell numbers ($p=0.0125$ in $CD4^+$ T cells and $p=0.0022$ in $CD8^+$ T cells) even after approximately 3 years of IFN-β administration; (2) the intracellular IFN-γ / IL-4 ratio tended to decrease in both $CD4^+$ and $CD8^+$ T cells ($p=0.0535$ and $p=0.0783$, respectively), reflecting a strong downmodulation of type 1 cytokine producing cells; and importantly (3) alterations such as the decreased intracellular IFN-γ / IL-4 ratio in $CD4^+$ T cells and increased percentage of $CD8^+$ $IL\text{-}13^+$ T cells compared with the pretreatment levels were only statistically significant in MS patients without relapse during IFN-β therapy ($p=0.0152$ and $p=0.0078$, respectively). Therefore, we consider that cytokine deviation toward the Th2 and Tc2 sides is linked to a long-term favorable response to IFN-β, while a higher intracellular IFN-γ / IL-4 ratio is associated with treatment failure.

Keywords: Multiple sclerosis; Interferon-β; Interferon-γ; Interleukin-4; Interleukin-13; Th1; Th2

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that is generally considered to be mediated by myelin-autoreactive T cells. Interferon beta (IFN-β) reduces the frequency and severity of clinical relapses in relapsing–remitting MS, and the basis of these beneficial effects has been extensively studied both in vivo and in vitro.

The effects of IFN-β on the cytokine production pattern is especially important, since increasing evidence suggests that MS is largely caused by $CD4^+$ T helper 1 (Th1) cells that produce interferon gamma (IFN-γ) but not interleukin (IL)-4 [1]. Moreover, important roles of $CD8^+$ T cells have also been suggested by the selective enrichment of memory $CD8^+$ T cells in the cerebrospinal fluid (CSF) of MS patients [2] together with diffuse infiltration of clonally expanded $CD8^+$ T cells into the brain parenchyma [3,4]. However, most previous cytokine studies on the effects of IFN-β have been performed within 1 year after initiation of the therapy [5–10], although the beneficial effects of IFN-β persist for

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several years [11]. Thus, although the long-term beneficial effects of IFN-β are well established, the basis of these effects in vivo remains to be fully elucidated. We recently performed sequential measurements of multiple cytokine production patterns in both $CD4^+$ and $CD8^+$ T cells from the pretreatment period up to 48 months after initiation of the therapy, and found early increases in Th2 cytokines, such as IL-4 and IL-13, followed by late decreases in Th1 cytokines, such as IFN-γ, in both conventional MS (C-MS) and opticospinal MS (OS-MS) [12]. In the present study, we focused on the cytokines that displayed significant alterations in our previous study and evaluated the long-term effects of IFN-β on the cytokine production patterns in $CD4^+$ and $CD8^+$ T cells in the peripheral blood of MS patients several years after initiation of the drug treatment.

2. Materials and methods

2.1. Patients

Fifteen Japanese patients (8 women and 7 men; mean age ± SD: 42.13 ± 12.44 years) with relapsing–remitting MS, diagnosed according to the revised diagnostic criteria for MS [13] and treated with IFN-β-1b (Betaferon®; Shering) 8×10^6 units given subcutaneously every other day for at least 2 years (mean period ± SD: 34.5 ± 5.5 months; range: 26–43 months), were included in this study. At the time of their enrollment, none of the patients were experiencing an acute attack or had been under immunosuppressive treatment for at least the previous 3 months. The patients were clinically classified into two subtypes: C-MS (13 patients) and OS-MS (2 patients), as described previously [14]. Briefly, patients who had both optic nerve and spinal cord involvement without any clinical evidence of disease in either the cerebrum or the cerebellum were considered to have OS-MS. Patients with minor brainstem signs, such as transient double vision or gaze nystagmus, were included in this subtype. All other patients showing disseminated involvement of the CNS were considered to have C-MS. The demographic features of the patients are shown in Table 1.

2.2. Intracellular cytokine analysis by flow cytometry

The intracellular cytokine patterns were studied by flow cytometry, as described previously [12]. IFN-γ was examined as a Th1 cytokine, while IL-4 and IL-13 were investigated as Th2 cytokines. Peripheral blood mononuclear cells were collected from the patients before treatment and after 2–3 years of IFN-β-1b therapy, and treated with 25 ng/ml phorbol 12-myristate 13-acetate (Sigma, St. Louis, MO), 1 μg/ml of ionomycin (Sigma) and 10 μg/ml brefeldin A (Sigma) for 4 h. The monoclonal antibodies used were: PC5-conjugated anti-CD4 (13B8.2; Becton Dickinson, San Jose, CA), PC5-conjugated anti-CD8 (B9.11; Becton Dickinson), FITC-conjugated anti-IFN-γ (25723.11; Becton Dickinson), PE-conjugated anti-IL-4 (3010.211; Becton Dickinson) and PE-conjugated anti-IL-13 (JES10-5A2; PharMingen, San Diego, CA). The percentages of cytokine-positive $CD4^+$ and $CD8^+$ cells were determined as the % cytokine-positive $CD4^+$ population/total $CD4^+$ population and the % cytokine-positive $CD8^+$ population/total $CD8^+$ population, respectively. Pretreatment measures of cytokine production were done twice at distinct time (average 3.1 days apart) in all MS patients and 9 of 15 in post-treatment measures and means were used when measured twice. According to the results of a preliminary study using 15 subjects, interassay variabilities were as follows; 14.2% in $CD4^+IL\text{-}4^+IFN\text{-}\gamma^-$ cell percentage, 11.6% in $CD4^+IL\text{-}4^-IFN\text{-}\gamma^+$ cell percentage, 29.0% in $CD4^+IL\text{-}13^+$ cell percentage, 13.8% in $CD8^+IL\text{-}4^+IFN\text{-}\gamma^-$ cell percentage, 28.2% in $CD8^+IL\text{-}4^-IFN\text{-}\gamma^+$ cell percentage, 42.1% in $CD8^+IL\text{-}13^+$ cell percentage.

2.3. Statistical analysis

Statistical analyses comparing age at baseline, disease duration at baseline and EDSS scores were performed by the Mann–Whitney *U*-test, while those for the gender ratio were carried out by the Fisher's exact probability test. Statistical analyses comparing the cell percentage and ratio of intracellular cytokine-producing $CD4^+$ and $CD8^+$ T cells between responders and non-responders were performed by the Mann–Whitney *U*-test, and those comparing the cell

Table 1
Demographic features of the 15 MS patients before and during IFN-β-1b treatment

	Total	Non-relapsed	Relapsed
Number of patients	15	9	6
Gender (male : female)	8 : 7	6 : 3	2 : 4
Age at baseline (mean ± S.D.)[a]	42.13 ± 12.44	40.33 ± 10.92	44.83 ± 15.09
Disease duration at baseline (mean ± S.D.)[a]	5.42 ± 3.64	4.30 ± 2.92	7.11 ± 4.22
EDSS at baseline (mean ± S.D.)	4.03 ± 2.18	3.94 ± 2.48	4.17 ± 1.86
EDSS after the observation period (mean ± S.D.)	4.27 ± 2.27	4.22 ± 2.59	4.33 ± 1.72
Relapse rate during the 2 years before IFN-β-1b (mean S.D.)	3.00 ± 2.24	2.56 ± 1.88	3.67 ± 2.73
Relapse rate during 1 year of IFN-β-1b (mean ± S.D.)	0.73 ± 1.38	0.00 ± 0.00	1.83 ± 1.72
Relapse rate during 1–2 years of IFN-β-1b (mean ± S.D.)	1.07 ± 1.67	0.00 ± 0.00	2.67 ± 1.63
Relapse rate during 2–3 years of IFN-β-1b (mean ± S.D.)	0.40 ± 0.63	0.00 ± 0.00	1.00 ± 0.63

[a] Years.

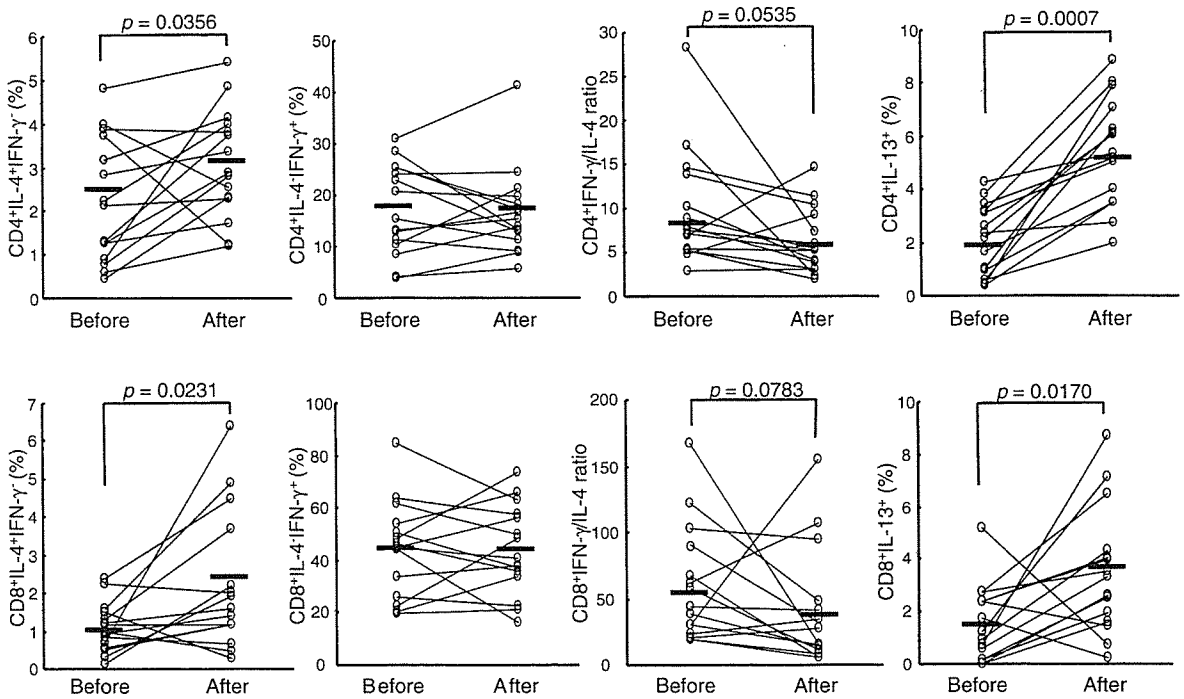

Fig. 1. Changes in the percentages and ratios of intracellular cytokine-producing $CD4^+$ (upper row) and $CD8^+$ (lower row) T cells before treatment and after 2–3 years of IFN-β-1b therapy. Data obtained from the same patient at different times are connected with a line. The bar indicates the mean value at each time point. The total number of MS patients is 15.

percentage and ratio of intracellular cytokine-producing $CD4^+$ and $CD8^+$ T cells between pre-treatment and after-treatment were performed by the Wilcoxon's rank test. A p value of less than 0.05 was considered significant.

3. Results

3.1. Clinical responses to IFN-β therapy

During the 2–3 year observation period, 6 patients suffered 33 relapses: 1 patient experienced 10 relapses, 1 experienced 8 relapses, 1 experienced 5 relapses, 2 experienced 4 relapses and 1 experienced 2 relapses. The remaining 9 patients did not have any relapses during the follow-up period. During this period, 5 of 13 (38%) C-MS patients and 1 of 2 (50%) OS-MS patients relapsed. The gender ratio, age at baseline, EDSS scores and annual relapse rate before initiation of the IFN-β-1b therapy did not differ significantly between the relapsed and non-relapsed patients.

3.2. Intracellular cytokines in $CD4^+$ T cells

In all 15 patients, the percentage of intracellular $CD4^+IL\text{-}4^+IFN\text{-}\gamma^-$ T cells was significantly increased after 2–3 years of IFN-β treatment compared with the pretreatment level

Table 2
Changes in the absolute numbers of intracellular cytokine-producing $CD4^+$ and $CD8^+$ T cells before treatment and after 2–3 years of IFN-β-1b therapy

	$CD4^+$			$CD8^+$		
	$IL\text{-}4^+IFN\text{-}\gamma^-$	$IL\text{-}4^-IFN\text{-}\gamma^+$	$IL\text{-}13^+$	$IL\text{-}4^+IFN\text{-}\gamma^-$	$IL\text{-}4^-IFN\text{-}\gamma^+$	$IL\text{-}13^+$
Before treatment	12.1±10.0	91.9±73.6	38.3±25.2	4.4±2.6	192.3±147.6	30.5±31.4
After treatment	9.5±7.5	50.2±56.2*	62.1±35.8*	4.4±4.8	88.1±65.4**	45.0±43.3 (/μl)
Non-relapsed						
Before treatment	14.4±10.8	92.5±59.7	41.5±25.2	5.0±3.0	163.5±87.3	32.7±32.8
After treatment	10.7±7.4	36.0±20.6*	70.9±32.9*	4.4±3.8	72.7±42.0**	55.0±48.3 (/μl)
Relapsed						
Before treatment	8.6±8.5	91.0±97.2	33.6±26.7	3.5±1.9	235.4±212.2	27.1±31.8
After treatment	7.7±8.0	71.6±85.1	49.0±38.8	4.4±6.5	111.1±90.1	29.9±32.5 (/μl)

* :$p<0.05$ (vs. before IFN-β-1b therapy).

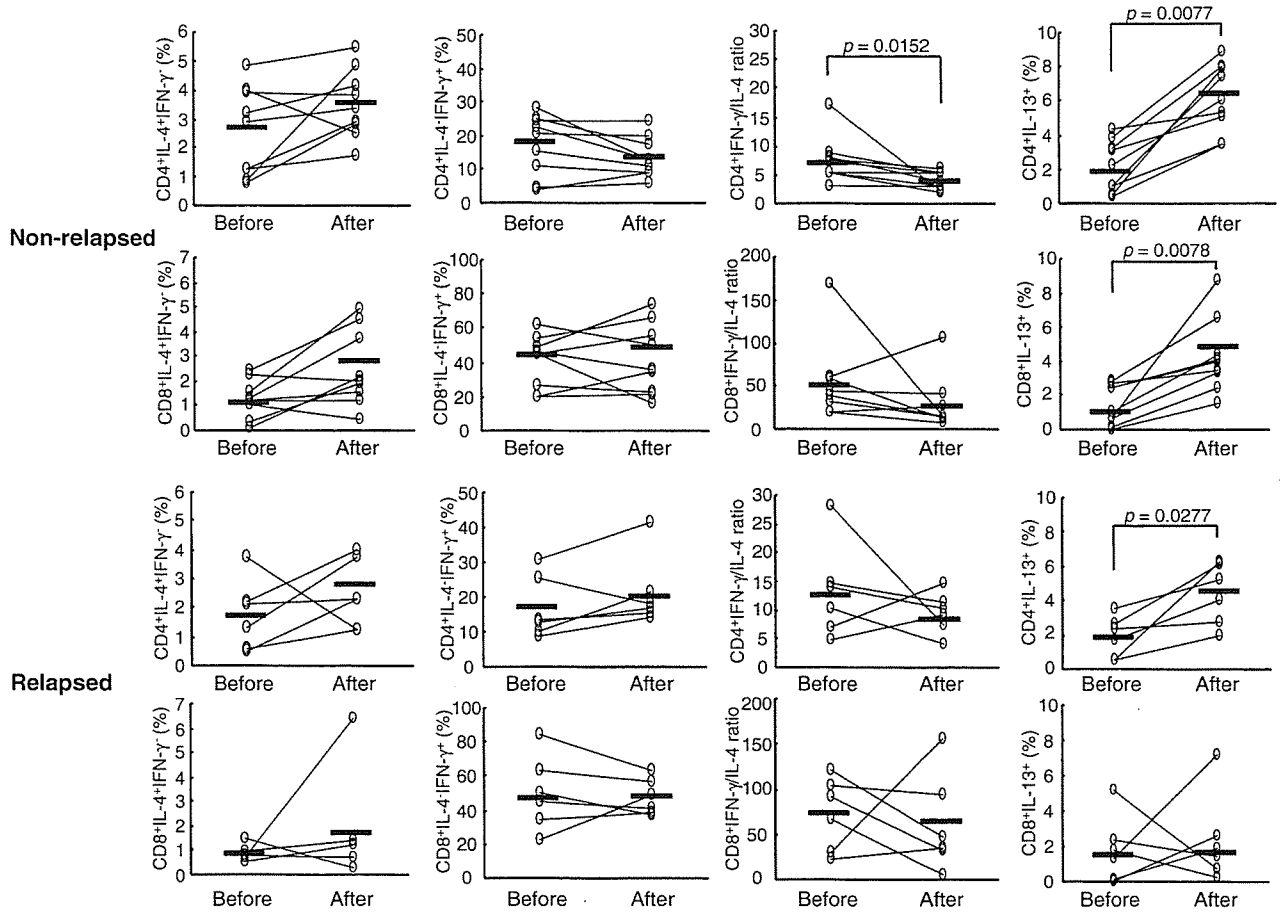

Fig. 2. Changes in the percentages and ratios of intracellular cytokine-producing $CD4^+$ and $CD8^+$ T cells according to the presence or absence of relapses during IFN-β-1b therapy. Data obtained from the same patient at different times are connected with a line. The bar indicates the mean value at each time point. There are 9 non-relapsed patients (upper panel) and 6 relapsed patients (lower panel).

($p=0.0356$) (Fig. 1). A similar significant augmentation pattern was observed for the percentage of intracellular $CD4^+IL\text{-}13^+$ T cells ($p=0.0007$). These changes were beyond the ranges of interassay variability (+ 62.8% in $CD4^+IL\text{-}4^+IFN\text{-}\gamma^-$ T cell percentages and + 165.3% in $CD4^+IL\text{-}13^+$ T cell percentage). The percentage of intracellular $CD4^+IL\text{-}4^-IFN\text{-}\gamma^+$ T cells did not change significantly with IFN-β treatment. Thus, the intracellular IFN-γ / IL-4 ratio tended to decrease after 2–3 years of treatment, but the decrease did not achieve statistical significance ($p=0.0535$) due to the small sample size. With respect to absolute cell numbers, decrease in $CD4^+IL4^-IFN\gamma^+$ T cells after treatment was statistically significant ($p=0.0125$) while $CD4^+IL4^+IFN\gamma^-$ T cells did not show a statistically significant changes (Table 2). In addition, $CD4^+IL\text{-}13^+$ T cells also increased significantly in an absolute number after treatment ($p=0.0231$).

3.3. Intracellular cytokines in $CD8^+$ T cells

In all 15 patients, the percentages of intracellular $CD8^+IL\text{-}4^+IFN\text{-}\gamma^-$ and $CD8^+IL\text{-}13^+$ T cells were significantly increased after 2–3 years of treatment compared with the pretreatment values ($p=0.0231$ and $p=0.0170$, respectively) (Fig. 1). These changes also exceeded the ranges of interassay variability (+130.4% in $CD8^+IL\text{-}4^+IFN\text{-}\gamma^-$ T cell percentages and +182.8% in $CD8^+IL\text{-}13^+$T cell percentage). The intracellular IFN-γ / IL-4 ratio tended to decrease after 2–3 years of treatment, but the decrease did not reach statistical significance ($p=0.0783$). Concerning absolute cell numbers, decrease in $CD8^+IL\text{-}4^-IFN\text{-}\gamma^+$ T cells after treatment was statistically significant ($p=0.0022$) while $CD8^+IL\text{-}4^+IFN\text{-}\gamma^-$ T cells did not show a significant change. Increase of $CD8^+IL\text{-}13^+$ T cells after treatment was not statistically significant in an absolute number.

3.4. Clinical responses and cytokine production patterns

The intracellular cytokine expression patterns were compared according to the clinical responses. MS patients without any relapses demonstrated significant decreases in the intracellular IFN-γ / IL-4 ratio in $CD4^+$T cells ($p=0.0152$) and increases in IL-13^+ cell percentages in both $CD4^+$ and $CD8^+$ T cells ($p=0.0077$ and $p=0.0078$,

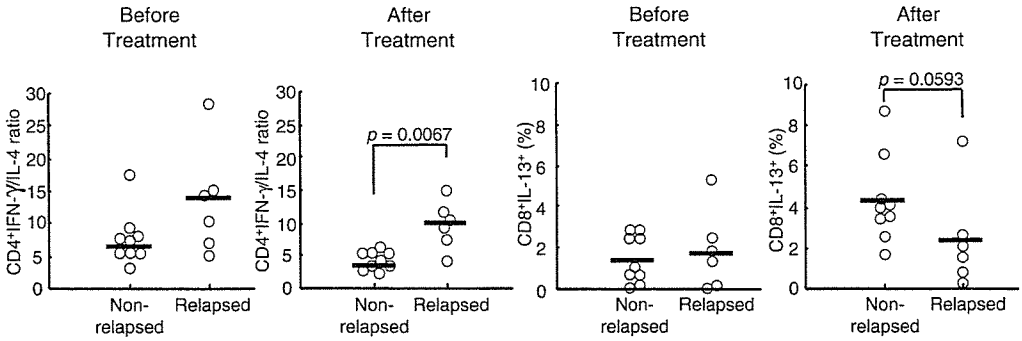

Fig. 3. Comparisons of the ratios of intracellular cytokine-producing $CD4^+$ T cells between relapsed and non-relapsed MS patients before treatment and after 2–3 years of IFN-β-1b therapy. The bar indicates the mean value in each group. There are 9 non-relapsed patients and 6 relapsed patients.

respectively) compared with the pretreatment levels, and patients who underwent relapses showed a significant increase in $CD4^+IL\text{-}13^+$ T cell percentage ($p=0.0277$) (Fig. 2). As to absolute cell numbers, both $CD4^+IL\text{-}4^-IFN\text{-}\gamma^+$ T cells and $CD8^+IL\text{-}4^-IFN\text{-}\gamma^+$ T cells decreased significantly after treatment only in non-relapsed patients ($p=0.0284$ and $p=0.0077$, respectively), but not in relapsed ones (Table 2). Absolute number increase in $CD4^+IL\text{-}13^+$ T cells was significant only in non-relapsed patients ($p=0.0209$) but not in relapsed patients while absolute number of $CD8^+IL\text{-}13^+$ T cells showed a tendency to increase after treatment in non-relapsed patients ($p=0.0858$) but not in relapsed ones.

When the intracellular cytokine expression patterns were compared between MS patients with and without relapses during IFN-β-1b therapy, the pretreatment values did not differ significantly between the two groups for any of the parameters (Fig. 3). However, after 2–3 years of treatment, the relapsed patients tended to have a lower percentage of $CD4^+IL\text{-}4^+IFN\text{-}\gamma^-$ T cells and a higher percentage of $CD4^+IL\text{-}4^-IFN\text{-}\gamma^+$ T cells than the non-relapsed patients ($p=0.0990$ and $p=0.0771$, respectively), resulting in a significantly higher intracellular IFN-γ / IL-4 ratio in $CD4^+$ T cells in the relapsed patients than in the non-relapsed patients ($p=0.0067$). On the other hand, after IFN-β treatment, the non-relapsed patients tended to have a higher percentage of $CD8^+IL\text{-}13^+$ T cells than the relapsed patients ($p=0.0593$). Concerning absolute cell numbers, there was no significant difference in any positive cells examined in either before or after treatment between the two groups (data not shown).

4. Discussion

This study revealed the following long-term effects of IFN-β on the intracellular cytokine production patterns of peripheral blood $CD4^+$ and $CD8^+$ T cells in MS patients: (1) type 2 cytokines, such as IL-4 and IL-13, were significantly increased in producing cell percentages in both $CD4^+$ and $CD8^+$ T cells while IFN-γ, a representative type 1 cytokine, was significantly decreased in the absolute producing cell numbers in both $CD4^+$ and $CD8^+$ T cells, even after approximately 3 years of IFN-β administration; (2) the intracellular IFN-γ / IL-4 ratio tended to decrease in both $CD4^+$ and $CD8^+$ T cells, reflecting a strong downmodulation of type 1 cytokines; and importantly (3) alterations such as the decreased intracellular IFN-γ / IL-4 ratio in $CD4^+$ T cells, increased percentage of $CD8^+IL\text{-}13^+$ T cells and increased cell numbers of $CD4^+IL\text{-}13^+$ T cells were only statistically significant in MS patients who responded well to IFN-β treatment, while the intracellular IFN-γ / IL-4 ratio in $CD4^+$ T cells was significantly higher in the relapsed patients than in the non-relapsed patients after long-term administration of IFN-β. Although each clinical parameter did not differ significantly between non-relapsed (responder) and relapsed (non-responder) patients, a longer disease duration, nevertheless higher relapse rate in the preceding two years before IFN-β initiation and resulting higher EDSS at baseline suggest high disease activity of the non-responders at baseline and could be a factor in the lack of response to IFN-β. Thus, difference in cytokine profile between responders and non-responders may reflect difference in baseline disease activity between the two groups.

Although the present study is an ex vivo study of peripheral blood lymphocytes after stimulation by PMA and ionomycin, the proportions of certain cytokine-producing cells obtained using this assay have been reported to reflect those destined to produce relevant cytokines in vivo [15]. Therefore, the results of the current study are considered to reflect the long-term in vivo effects of IFN-β on the Th1 / Th2 and Tc1 / Tc2 balance in MS.

Various mechanisms have been proposed to explain the beneficial effects of IFN-β treatment for MS. Among these, cytokine alterations probably play key roles, since cytokines are crucial for the induction and maintenance of autoimmune inflammation. Previous studies on the cytokine balance in MS under IFN-β treatment have reported the following conflicting findings: IFN-β-1a decreases IL-12 and augments IL-10 production [7]; IFN-β-1b decreases IFN-γ while IFN-β-1a enhances IL-4 and IL-10 [10]; IFN-β-1a [8] and IFN-β-1b [6] down-modulate both IFN-γ and IL-4 [6]; IFN-β-1b induces no changes in either Th1 or Th2 marker genes [9]; IFN-β-1b increases IFN-γ [5]. These

inconsistent results for the cytokine changes induced by IFN-β in MS probably reflect differences in the methodologies employed and distinct time points examined as well as the complex biology of the cytokines themselves.

To the best of our knowledge, there are no reports in the literature regarding the cytokine changes induced by administration of IFN-β for as long as 3 years. Moreover, cytokine profiles that can discriminate between favorable and unfavorable responses to long-term administration of IFN-β have not been clarified in detail. Although the number of MS patients who were successfully followed-up in the present study was not large, we found a significant decrease in the production of type 1 cytokine, IFN-γ, and relative increase in the production of type 2 cytokines, such as IL-4 and IL-13, in both $CD4^+$ and $CD8^+$ T cells, even after long-term treatment with IFN-β, suggesting that shift towards of Th2 and Tc2 sides is one of the major in vivo effects of IFN-β. On the other hand, an increase in type 1 cytokines, such as IFN-γ, was observed in a fraction of MS patients in the first few weeks in our previous study [12] and in the first 2 months by Dayal et al. [5], and this was not related to an increased number of relapses. However, in the late stage of IFN-β therapy, the present study revealed a significant difference in the intracellular IFN-γ / IL-4 ratio between relapsed and non-relapsed patients, with a higher ratio being associated with relapses. Therefore, a down-modulation of Th1 response with relative increase of Th2 response, which lowers the intracellular IFN-γ / IL-4 ratio, is considered to be critical for maintaining a relapse-free state under long-term IFN-β therapy.

Concerning $IL\text{-}13^+$ cells, their increase among $CD4^+$ T cells and $CD8^+$ T cells after long-term IFN-β administration was more pronounced in the non-relapsed patients than in the relapsed patients. These observations may indicate that a favorable response to IFN-β is linked to an elevation of $CD4^+IL\text{-}13^+$ T cells and $CD8^+IL\text{-}13^+$ T cells. We previously reported unexpected increases in $CD4^+IL\text{-}13^+$ and $CD8^+IL\text{-}13^+$ T cells at relapse in MS, especially in C-MS [16], the roles for which required clarification by further studies. The results of the present study suggest that these $IL\text{-}13^+$ cells may have a protective role, since the increase in $IL\text{-}13^+$ cells was associated with a decrease in the relapse rate. Therefore, the increase in these cells observed at relapse may be the host's efforts to overcome Th1-mediated inflammatory processes. The recent observation that $CD8^+$ T cells producing IL-13 were correlated negatively with T1 lesion loads on brain magnetic resonance imaging in MS [17] further suggests a role of $CD8^+$ suppressor cells in down-regulating the disease activity, and supports a protective role for $CD8^+IL\text{-}13^+$ T cells. IL-13 shares some receptor components with IL-4 and demonstrates similar functions to IL-4 [18]. IL-4 and IL-13 mostly act synergistically in the down-regulation of Th1 cells, although increasing evidence indicates that IL-13 rather than IL-4 plays a crucial role in many aspects of immune regulation. For example, IL-13 is essential for tolerance induction [19], renders T cells unresponsive to IL-12 [20] and decreases TNF-α-mediated cytotoxicity [21]. Although the mechanism of the protective action of IL-13 in MS remains to be clarified, an increase in the number of $IL\text{-}13^+$ cells could be one of the biomarkers for IFN-β responsiveness in MS.

In our previous study, similar cytokine changes during IFN-β therapy were noted in both OS-MS and C-MS [12] and it was suggested that the IFN-β action may have a common mechanism in the two MS subtypes, at least in the early course of the treatment. In the present study, the number of OS-MS patients was limited and further studies in a larger scale are necessary to address subtype-specific changes in the late phase of IFN-β therapy.

In summary, the results of the current study have revealed that alterations in the cytokine balance, such as a decreased intracellular IFN-γ / IL-4 ratio in $CD4^+$ T cells and an increase in $CD4^+IL\text{-}13^+$ T cells and $CD8^+IL\text{-}13^+$ T cells, are linked with a favorable response to IFN-β, while a higher intracellular IFN-γ / IL-4 ratio is associated with treatment failure after long-term IFN-β therapy in MS.

Acknowledgements

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Immunization with heat shock protein 105-pulsed dendritic cells leads to tumor rejection in mice ☆

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Abstract

Recently, we reported that heat shock protein 105 (HSP105) DNA vaccination induced anti-tumor immunity. In this study, we set up a preclinical study to investigate the usefulness of dendritic cells (DCs) pulsed with mouse HSP105 as a whole protein for cancer immunotherapy in vivo. The recombinant HSP105 did not induce DC maturation, and the mice vaccinated with HSP105-pulsed BM-DCs were markedly prevented from the growth of subcutaneous tumors, accompanied with a massive infiltration of both $CD4^+$ T cells and $CD8^+$ T cells into the tumors. In depletion experiments, we proved that both $CD4^+$ T cells and $CD8^+$ T cells play a crucial role in anti-tumor immunity. Both $CD4^+$ T cells and $CD8^+$ T cells specific to HSP105 were induced by stimulation with HSP105-pulsed DCs. As a result, vaccination of mice with BM-DCs pulsed with HSP105 itself could elicit a stronger tumor rejection in comparison to DNA vaccination.

Keywords: Heat shock protein 105; Cancer antigen; Dendritic cells; Th; CTL

Heat shock proteins (HSPs) are soluble intracellular proteins, which are ubiquitously expressed, and their expression can be induced at much higher levels as a result of heat shock or other forms of stress. HSPs have essential functions in the regulation of protein folding, conformation, assembly, and sorting. HSPs have been shown to be molecular chaperones that function to maintain the native conformational states of proteins and prevent protein–protein aggregation [1]. HSPs can also induce the response of antigen-specific effector $CD8^+$ T cells which can protect hosts from both infection and tumor challenge [2]. Srivastava and co-workers [3,4] led to a proposal that the tumor-derived HSP–peptide complex elicits a protective immunity that is specific to a particular cancer, while HSPs derived from normal tissues did not elicit any protective immunity to the cancers tested. Immunotherapeutic clinical trials targeted at autologous tumor-derived gp96–peptide complexes are still ongoing in metastatic melanoma and colorectal carcinoma patients [5].

Dendritic cells (DCs) are powerful antigen-presenting cells (APCs) that are considered to be potent immunotherapeutic agents to promote the host immune response against tumor antigen. DCs become efficient tumor vaccines when pulsed with synthetic or natural tumor-derived peptides, transduced with tumor-derived RNA or vectors

☆ *Abbreviations:* BM-DC, bone marrow-derived DC; HSP105, heat shock protein 105; Th, helper T cell; CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; C26 (C20), colon 26 clone 20.

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encoding for tumor-associated proteins, or directly fused to or incubated with tumor cells [6]. For effective induction of cytotoxic T lymphocytes (CTLs) by vaccination, "Cross-presentation" mediated by DCs often plays an important role. Such cross-presentation includes the antigen presentation of exogenous antigens by major histocompatibility complex (MHC) class I molecules as well as by MHC class II molecules [7,8]. HSP-chaperoned peptides were cross-presented by the MHC class I molecules of the DCs several 100-fold more efficiently than unchaperoned peptides [9]. In addition, CD91, also called α_2-macroglobulin receptor is expressed on DCs and has been shown to act as one of the receptors for HSP-chaperoned peptides to efficiently incorporate the HSP–peptide complexes [10].

We earlier reported that heat shock protein 105 (HSP105) was overexpressed in a variety of human cancers but it is not expressed in normal tissue except for the testes [11,12], thus suggesting that HSP105 itself may be a potential candidate as a target antigen for cancer immunotherapy. The amino acid sequences and expression patterns of HSP105 are very similar between humans and mice. HSP105 has been found to be immunogenic in mice and an effective anti-tumor immunity has been observed after *HSP105* DNA vaccination [13]. In the present study, we set up a preclinical study to investigate the usefulness of HSP105 as a target for cancer immunotherapy using DCs. It has been reported that HSPs can induce DC maturation and activation as determined by the upregulation of MHC class II and CD86 molecules, the secretion of IL-12 and TNFα [14,15], and migration into draining lymphoid organs [16]. On the contrary, some investigators reported that HSP-mediated maturation of DCs was caused by contaminating lipopolysaccharide (LPS) fraction because endotoxin-free HSP70 failed to induce DC maturation [17]. We herein show that the highly purified HSP105 did not induce DC maturation and that the immunization of HSP105-pulsed DC led to the tumor rejection of melanoma and colorectal cancer in mice. These findings suggested that HSP105 itself could be a valuable tumor-associated antigen applicable for DC-based immunotherapy of tumors overexpressing it.

Materials and methods

Cell lines and mice. A subline of BALB/c-derived colorectal cancer cell line Colon 26, C26 (C20), was provided by Dr. Kyoichi Shimomura (Astellas Pharmaceutical Co., Tsukuba, Japan). Other cancer cell lines were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan). All these cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum at 37 °C in a humidified 5% CO_2 atmosphere. We used the B16-F10 melanoma cell line syngeneic to C57BL/6 mice and C26 (C20) for the tumor challenge. Female 6- to 8-week-old C57BL/6 mice (H-2^b) and BALB/c mice (H-2^d) were purchased from Charles River Japan (Yokohama, Japan). These mice were kept under specific pathogen-free conditions. These experiments were approved by the Animal Research Committee of Kumamoto University.

Production of recombinant proteins. We produced highly purified recombinant mouse HSP105 from the *Escherichia coli* strain BL21 cells transduced with the mouse *HSP105* gene expression vector, as described previously [18]. Purified proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and Coomassie brilliant blue (CBB)-stained bands were quantified by densitometry. Thereafter, by using affinity chromatography on a polymyxin B agarose gel (Sigma Chemical Co., St. Louis, MO), the endotoxin levels were decreased. We also produced highly purified recombinant myelin basic protein (MBP) as described previously [19]. Both recombinant HSP105 and MBP were estimated to be almost endotoxin free by using Limulus amebocyte lysate assay kit (BioWhittaker, Walkersville, MD), and endotoxin contents in these two materials were below 10 endotoxin U/mg.

Immunizations and tumor challenge. Bone marrow-derived DCs (BM-DC) were prepared as described previously [20]. BM-DCs were pulsed with 2 μg/ml HSP105 at 37 °C for 16 h, non-adherent and loosely adherent proliferating DCs were collected and used as HSP105-pulsed BM-DC. In tumor prevention experiments, mice were intraperitoneally inoculated with HSP105-pulsed BM-DC (5×10^5) suspended in 200 μl PBS on days −14 and −7. In parallel, groups of mice were injected with BM-DC alone or PBS as controls. Tumor challenge was initiated by subcutaneous injection with B16-F10 cells (1×10^4) or C26 (C20) cells (3×10^4) suspended in 100 μl HBSS (Gibco, Grand Island, NY) in shaved right flanks on day 0. Tumor occurrence was observed twice a week. The tumor size was evaluated by measuring two perpendicular diameters using calipers.

Flow cytometric analysis. Staining of cells and analysis on a flow cytometer (FACScan; BD Biosciences) were done as described previously [21]. Antibodies and reagents used for staining were as follows: FITC-conjugated anti-I-A^b (clone 28-16-8S; mouse IgG2a; Caltag, Burlingame, CA), R-PE-conjugated anti-mouse CD80 (clone RMMP-1; rat IgG2a; Caltag), R-PE-conjugated anti-mouse CD86 (clone RMMP-2; rat IgG2a; Caltag), FITC-conjugated anti-mouse CD4 (clone L3T4; rat IgG2a; BD PharMingen, San Diego, CA), FITC-conjugated anti-mouse CD8 (clone Ly-2; rat IgG2a; BD PharMingen), FITC-conjugated mouse IgG2a control (clone G155-178; BD PharMingen), and R-PE-conjugated rat IgG2a control (clone LO-DNP-16; Caltag).

Depletion of $CD4^+$ T cells and $CD8^+$ T cells in mice. Rat monoclonal antibodies (mAbs) GK1.5 specific to mouse CD4 and 2.43 specific to mouse CD8 were used to deplete $CD4^+$ T cells and $CD8^+$ T cells in vivo, respectively. The mice were injected with ascites (0.1 ml/mouse) from hybridoma-bearing nude mice intraperitoneally on days −18, −15, −11, −8, −4, and −1 and the tumor cells were inoculated on day 0. Normal rat IgG (Sigma, St. Louis, MO; 200 μg/mouse) was used as a control. The depletion of T cell subsets was monitored by a flow cytometric analysis, which showed more than a 90% specific depletion in the number of splenocytes.

Immunohistochemical analysis. Immunohistochemical detection of HSP105 was done as previously described [11,12]. Rabbit polyclonal anti-human HSP105 (Santa Cruz, Santa Cruz, CA) was used as the primary antibody in this study. Immunohistochemical staining of CD4 and CD8 was done as previously described [22]. For the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) method, we used ApopTag Fluorescein In Situ Apoptosis Detection Kits (Serologicals Corporation, Norcross, GA).

Induction of $CD4^+$ T cells and $CD8^+$ T cells specific to HSP105. The mice were inoculated intraperitoneally with HSP105-pulsed BM-DC on days −14 and −7. Spleen cells were harvested on day 0, and $CD4^+$ T cells and $CD8^+$ T cells were purified using the magnetic cell sorting system (MACS) with anti-mouse CD4 (L3T4) mAb and anti-mouse CD8α (Ly-2) mAb, respectively. The purity of these T cell subsets exceeded 95% by a flow cytometric analysis. $CD4^+$ T cells or $CD8^+$ T cells (3×10^5/well) were separately incubated in RPMI 1640 medium supplemented with 10% horse serum, IL-2 (100 U/ml), and 2-ME (50 μM) together with the irradiated (4500 Gy) HSP105-pulsed BM-DC in 24-well culture plates. BM-DCs (3×10^4/well) pulsed with 2 μg/ml HSP105 for 16 h were irradiated (4500 Gy) and added to the culture wells for the restimulation once a week. After the third restimulation in vitro, both proliferation and cytotoxicity assays were performed as described previously [23]. For the

control of ^{51}Cr-release assay, $CD8^+$ T cells isolated from the mice immunized with BM-DCs alone were restimulated in vitro with BM-DCs alone once a week and used as effector cells.

ELISPOT assay. HSP105-specific IFN-γ production of T cells was quantified using the appropriate ELISPOT kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. $CD4^+$ T cells or $CD8^+$ T cells were incubated with the BM-DC alone, BM-DCs pre-pulsed with HSP105, or BM-DCs pre-pulsed with myelin basic protein (MBP) as a control at 37 °C for 24 h. Each BM-DC was pre-pulsed with 2 μg/ml protein at 37 °C for 16 h. The spots were automatically counted and subsequently analyzed using the Eliphoto system (MINERVA TECH, Tokyo, Japan).

Statistical analysis. The statistical significance of the differences in the findings between the experimental groups was determined by Student's *t* test. The overall survival rate was calculated using the Kaplan–Meier method, and statistical significance was evaluated using Wilcoxon's test. A value of $P < 0.05$ was considered to be statistically significant.

Results

HSP 105 does not induce maturation of DCs

To analyze the direct effect of HSP 105 used in this study on BM-DCs, BM-DCs were incubated with HSP105, LPS as a positive control, and left untreated for 16 h. As shown in Fig. 1, no significant difference was observed in the levels of surface expression of CD80, CD86, and I-A^b between untreated BM-DCs and HSP105-pulsed BM-DCs. Moreover, HSP105-pulsed BM-DCs microscopically did not show any morphological changes in comparison to the untreated BM-DC. On the contrary, LPS-pulsed BM-DCs exhibited markedly increased expression of these three molecules. Although it is reported that HSPs could induce DC maturation and activation [14–16], the recombinant HSP105 used in this study including little LPS (below 10 endotoxin U/mg) did not show such activity. Thereafter, we evaluated the antigenicity of HSP105 to induce anti-tumor immunity.

The HSP105-pulsed BM-DC vaccine induced anti-tumor immunity against the lethal challenge of B16-F10 and C26 (C20)

We recently reported that mouse HSP105 was also overexpressed in the liver metastasis of C26 (C20) cells, and lung metastase of the B16-F10 cells, and that *HSP105* DNA vaccination inhibited the growth of these tumors [13]. In this study, we investigated the effects of HSP105 vaccination based on DCs on the growth of B16-F10 and C26 (C20) tumor cells in vivo. The objective was to determine whether prophylactic vaccination induced significant immunity against tumor growth and a prolonged survival. The protocol of vaccination in this study is shown in Fig. 2A. The results shown in Fig. 2B demonstrate that immunization with HSP105-pulsed BM-DC markedly inhibited the growth of B16-F10 tumors in comparison to other groups ($P < 0.01$). As shown in Fig. 2C, five of eight (62.5%) mice immunized with HSP105-pulsed BM-DC remained tumor free and survived for 100 days after the tumor challenge. In contrast, the mice vaccinated with BM-DC alone (12.5%) or PBS (0%) showed little protection against the growth of B16-F10 tumor in comparison to the observations in mice treated with HSP105-pulsed

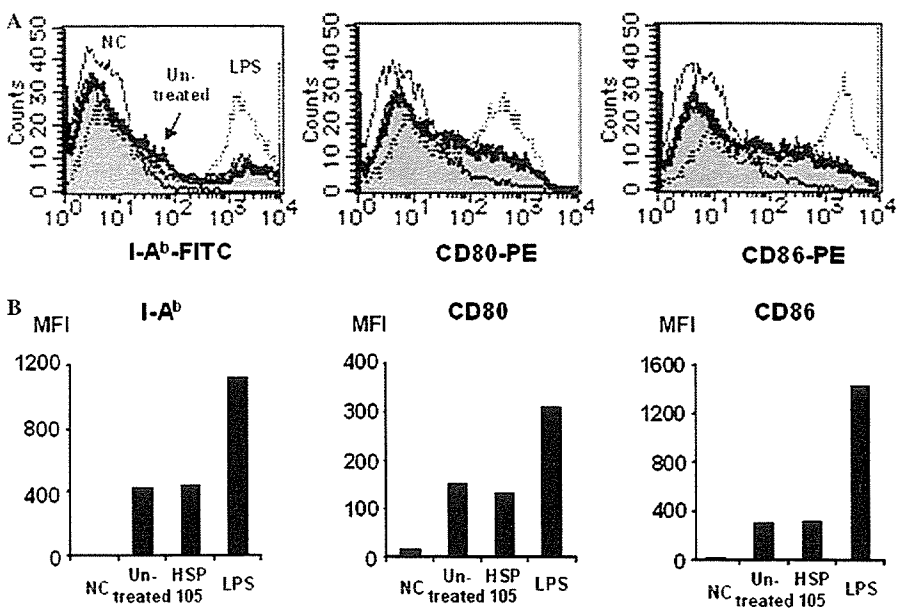

Fig. 1. Expression levels of cell surface I-A^b, CD80, and CD86 on BM-DCs, HSP105-pulsed BM-DCs, and LPS-pulsed BM-DCs were analyzed by flow cytometric analysis. BM-DCs were pulsed with 2 μg/ml HSP105, 1 μg/ml LPS or left untreated for 16 h. (A) The expression levels in HSP105-pulsed BM-DCs (filled histogram), LPS-pulsed BM-DCs (dotted line), and untreated BM-DCs (thick line), and the profiles of cells treated with isotype matched Ig as a negative control for staining (thin line). (B) The mean fluorescence intensity (MFI) of I-A^b, CD80, and CD86 staining in the cells. The results are representative of three independent experiments with similar results.

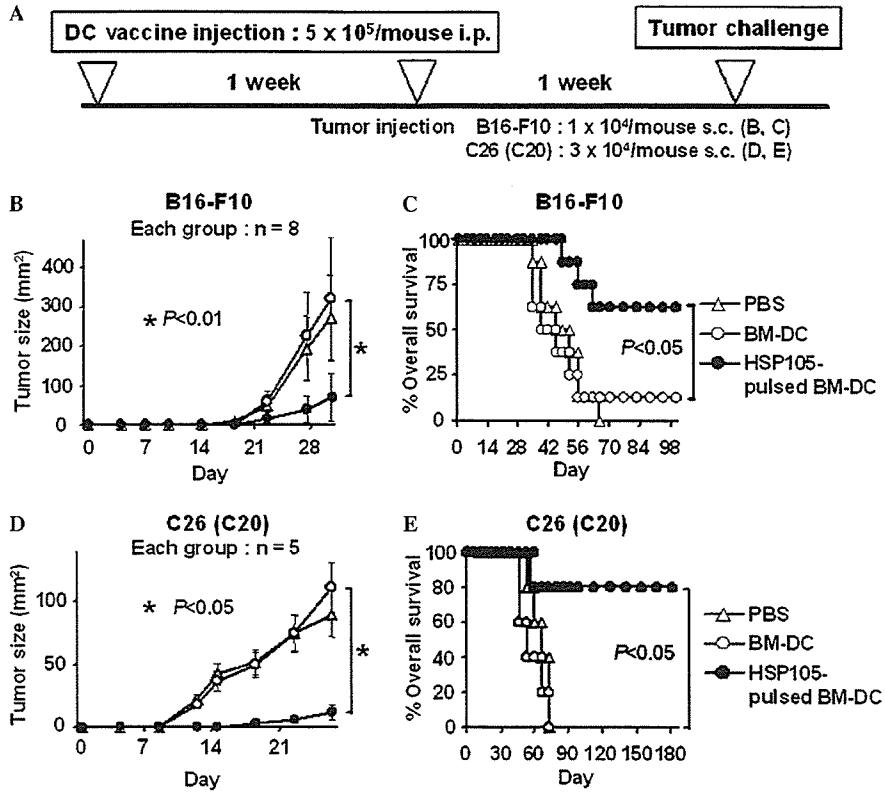

Fig. 2. Protection against tumor growth of B16-F10 and C26 (C20) cells by immunization with HSP105-pulsed BM-DC vaccine. (A) Protocol of the vaccination. The mice were immunized with PBS, BM-DC alone, and HSP105-pulsed BM-DC on 14 and 7 days before the tumor challenge. Seven days after the second immunization, the mice were challenged with B16-F10 cells s.c. (B,C), or C26 (C20) cells s.c. (D,E). (B,D) The tumor size was evaluated by measuring two perpendicular diameters. The result is presented as the mean area of tumor ± SE, and we evaluated statistical significance of the differences between each group using the unpaired Student's *t* test. (C,E) The mice in each group were observed for their survival period. The statistical significance of the differences between each group was evaluated using Wilcoxon's test.

BM-DC ($P < 0.05$). Similar results were observed in a prophylactic immunotherapy model using C26 (C20). Four of five (80%) mice immunized with HSP105-pulsed BM-DC completely rejected the C26 (C20) (3×10^4) cells (Figs. 2D and E), whereas tumors grew rapidly and all five mice died within 70 days in control mice treated with PBS or BM-DC alone. These results suggest that the HSP105-pulsed BM-DC vaccine is a potent vaccine that can efficiently induce specific anti-tumor immunity.

Both $CD4^+$ T cells and $CD8^+$ T cells are required for anti-tumor immunity

To determine the role of $CD4^+$ T cells and $CD8^+$ T cells in the protection against B16-F10 and C26 (C20) tumor cells induced by HSP105 vaccination, we depleted mice of $CD4^+$ T cells or $CD8^+$ T cells by the treatment with anti-CD4 or anti-CD8 mAb in vivo, respectively. During the depletion procedure, the mice were immunized with HSP105-pulsed BM-DC vaccine and challenged with B16-F10 or C26 (C20) cells (Fig. 3A). In both B16-F10 and C26 (C20) models, mice depleted of $CD4^+$ T cells, and $CD8^+$ T cells developed aggressively growing tumors after the challenge in comparison to the findings in control mice treated with rat IgG ($P < 0.05$) (Figs. 3B and D). The mice depleted of $CD4^+$ T cells or $CD8^+$ T cells all died by 52–65 days, whereas more than 50% of the control mice survived for 70 days ($P < 0.05$) (Figs. 3C and E). These results suggest that both $CD4^+$ T cells and $CD8^+$ T cells play crucial roles in the protective anti-tumor immunity induced by the HSP105-pulsed BM-DC vaccine.

Vaccination of HSP105-pulsed BM-DCs induced infiltrations of both $CD4^+$ T cells and $CD8^+$ T cells into tumor cells, but not into normal organs

Four of five (80%) mice immunized with the HSP105-pulsed BM-DCs completely rejected challenges of C26 (C20) cells (3×10^4) (Fig. 2E). To ascertain whether these rejections were induced by $CD4^+$ T cells or $CD8^+$ T cells, the subcutaneous inoculation of many C26 (C20) cells (1×10^6) into the right flank was done at 7 days after the second vaccination. After tumor formation, we removed the tumor and immunohistochemically stained it using anti-CD4 mAb, anti-CD8 mAb, and the TUNEL method. The infiltration of $CD4^+$ T cells and $CD8^+$ T cells into C26

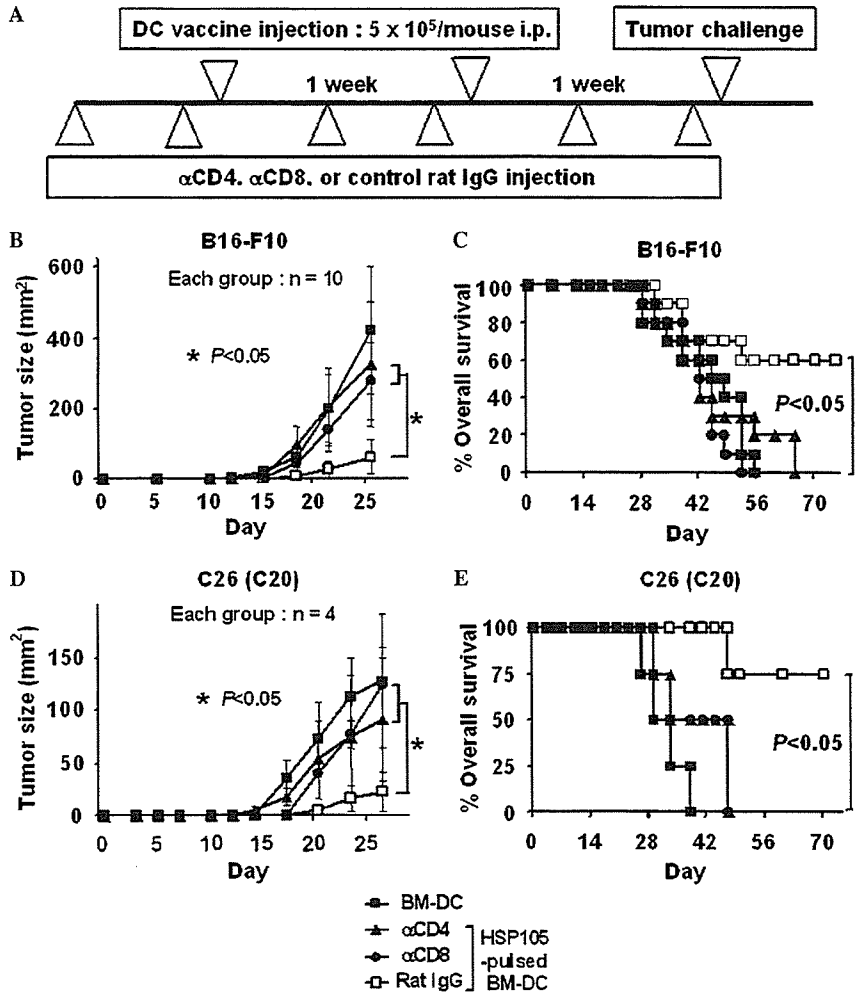

Fig. 3. Both $CD4^+$ and $CD8^+$ T cells are involved in the antitumor immunity elicited by the HSP105-pulsed DC vaccine. (A) Protocol for the vaccination and the depletion of T cells. C57BL/6 mice and BALB/c mice were challenged s.c. with B16-F10 cells and C26 (C20) cells, respectively. (B,D) The tumor size was evaluated by measuring two perpendicular diameters. The result is presented as the mean area of tumor ± SE, and we evaluated the statistical significance of the differences between each group using the unpaired Student's *t* test. (C,E) The mice in each group were observed for their survival period. The statistical significance of the differences between each group was evaluated using Wilcoxon's test.

(C20) tumors and some apoptotic C26 (C20) tumor cells were observed in the mice vaccinated with HSP105-pulsed BM-DCs, but never in the mice vaccinated with unpulsed BM-DCs (Fig. 4A). These results suggest that HSP105-pulsed BM-DCs have the potential to sensitize many HSP105-specific $CD4^+$ T cells and $CD8^+$ T cells to kill C26 (C20) tumor cells.

We evaluated the risk of autoimmunity by immunization against self-HSP105. Both BALB/c and C57BL/6 mice immunized with HSP105-pulsed BM-DC were apparently healthy without any abnormality such as dermatitis, arthritis, or neurological disorders. The tissues of the mice immunized with HSP105-pulsed BM-DC were histologically examined. The brain, liver, heart, kidneys, and spleen had normal structures and did not show any pathological changes suggestive of an immune response, such as the infiltration of $CD4^+$ T cells and $CD8^+$ T cells or tissue destruction and repair. Although we used female mice for the experiments described above, we also immunized male mice with HSP105-pulsed BM-DC to ascertain whether immunization with HSP105-pulsed BM-DC induced autoimmunity in the testis in which HSP105 is strongly expressed. However, no sign of autoimmunity was observed in the testis (Fig. 4B).

Induction of HSP105-specific $CD4^+$ T cells and $CD8^+$ T cells by immunization with HSP105-pulsed BM-DC

$CD4^+$ T cell lines specific to HSP105 were established from spleen cells derived from mice vaccinated with HSP105-pulsed BM-DC. $CD4^+$ T cells were separated from spleen cells and the purity of these cells was more than 95% by flow cytometric analysis. These cells were restimulated with irradiated and HSP105-pulsed DCs once

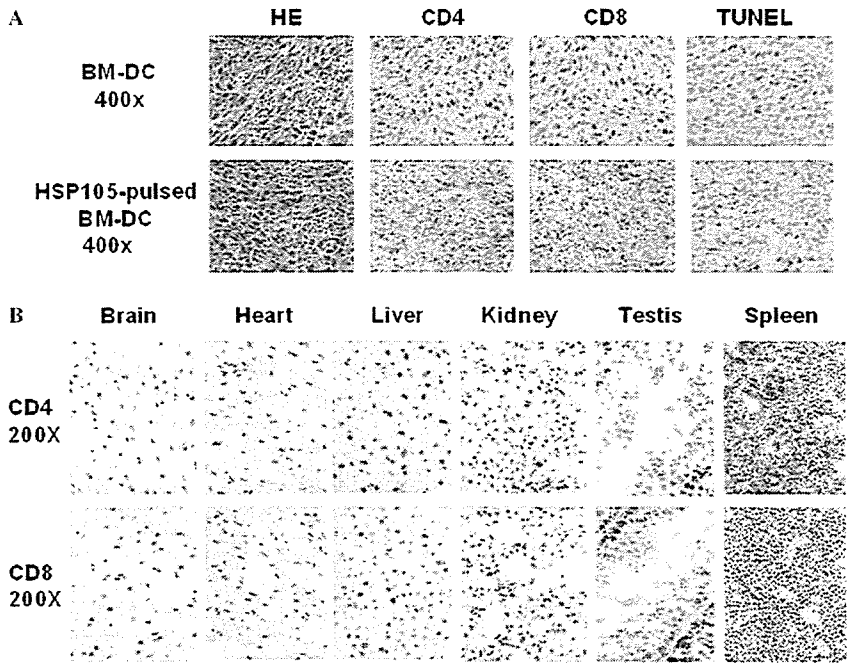

Fig. 4. Vaccination of HSP105-pulsed BM-DCs induced infiltrations of both $CD4^+$ T cells and $CD8^+$ T cells into C26 (C20) tumor and induced the apoptosis of C26 (C20) tumor cells. (A) C26 tumors removed from the mice vaccinated with BM-DCs or HSP105-pulsed BM-DCs were analyzed using immunohistochemical staining with anti-CD4 mAb, anti-CD8 mAb, and the TUNEL method on 4 days after the inoculation of tumor cells (1×10^6). (B) Normal tissue specimens of mice vaccinated with HSP105-pulsed BM-DCs were examined histologically and immunohistochemically. Objective magnification was 200×. The spleen was used as a positive control for the staining of both $CD4^+$ and $CD8^+$ cells.

a week. After three restimulations, both an ELISPOT assay and a proliferation assay were performed. The ELISPOT assay showed that HSP105-sensitized $CD4^+$ T cells produced IFN-γ in response to BM-DCs prepulsed with HSP105 but not an irrelevant MBP (Fig. 5A). As shown in Fig. 5B, HSP105-sensitized $CD4^+$ T cells proliferated in the presence of BM-DCs prepulsed with HSP105 but not MBP. These observations clearly indicated that HSP105-specific $CD4^+$ T cells were included in the T cell line.

We investigated whether HSP105-specific $CD8^+$ T cells were also induced with HSP105-pulsed DC vaccination. $CD8^+$ T cells were purified (>95%) from spleen cells of vaccinated mice and restimulated with irradiated and HSP105-pulsed DCs once a week. After three restimulations, the ELISPOT assay and 6 h ^{51}Cr-release assay were performed to detect the HSP105-specific CTL responses (Figs. 5C and D). The $CD8^+$ T cell line exhibited a HSP105-specific production of IFN-γ in an ELISPOT assay when cells were stimulated with BM-DCs prepulsed with HSP105 but not MBP ($P < 0.01$), however, the number of spots was smaller than that of $CD4^+$ T cells (Fig. 5C). $CD8^+$ T cells immunized with HSP 105-pulsed DC demonstrated a significant cytolytic activity against the B16-F10 cells pretreated with IFN-γ to induce the expression of MHC class I molecules on the cell surface, whereas $CD8^+$ T cells from mice immunized with BM-DC alone revealed little cytolytic activity ($P < 0.005$) (Fig. 5D). The induction of HSP105-specific $CD8^+$ T cells by the immunization in vivo with HSP105-pulsed BM-DC and the stimulation of the $CD8^+$ T cell line in vitro with the HSP105-pulsed BM-DC strongly suggested that these HSP105-specific $CD8^+$ T cells were induced by the cross-presentation of HSP105 by BM-DCs.

Discussion

HSPs are classified into several families based on their apparent molecular weights, such as HSP105/110, HSP90, HSP70, HSP60, HSP40, and HSP27 [24]. HSP105 consists of HSP105α and HSP105β. HSP105α is a constitutively expressed 105-kDa HSP that is induced by a variety of stresses, whereas HSP105β is a 90-kDa truncated form of HSP105α that is specifically induced by heat shock at 42 °C [24]. In this study, we used the mouse HSP105α protein. The cDNA sequence of murine HSP105 is almost the same as that of the Chinese hamster HSP110 [25,26], so HSP105 belongs to a member of the HSP105/110 family. We recently reported by the immunohistochemical analysis that HSP105 is overexpressed in a variety of human tumors [12], the liver metastasis of the C26 (C20) cells in the BALB/c mice, and lung metastasis of the B16-F10 cells in the C57BL/6 mice [13]. We examined the expression of HSP105 in the mouse cancer cell lines using a Western blotting analysis and found that HSP105 was strongly expressed in all 7 mouse cell lines tested (data not shown).

Many studies have shown that certain HSPs purified from a tumor can function as an effective vaccine against the same

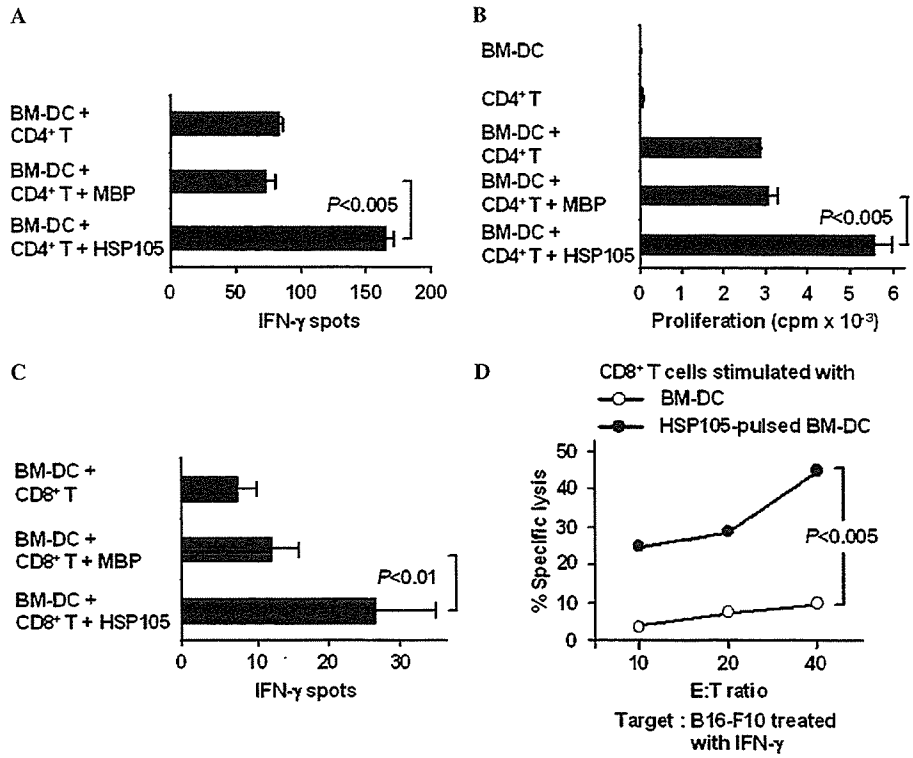

Fig. 5. Induction of HSP105-specific $CD4^+$ T cells and $CD8^+$ T cells by stimulation with HSP105-pulsed BM-DCs. (A) An ELISPOT assay for IFN-γ production by $CD4^+$ T cell lines stimulated with HSP105 protein-pulsed BM-DCs. $CD4^+$ T cells derived from the mice vaccinated with HSP105-pulsed BM-DC were stimulated in vitro with HSP105-pulsed BM-DC three times. For the ELISPOT assay, these $CD4^+$ T cells were co-cultured with BM-DC prepulsed with HSP105, MBP, or unpulsed BM-DC for 24 h. (B) Cell proliferation of $CD4^+$ T cell lines stimulated with HSP105-pulsed BM-DCs was determined by measuring [^{3}H]thymidine incorporation. $CD4^+$ T cells were co-cultured with BM-DC prepulsed with HSP105, MBP, or unpulsed BM-DC for 72 h. (C) An ELISPOT assay for IFN-γ production by $CD8^+$ T cell lines stimulated with HSP105-pulsed BM-DCs. $CD8^+$ T cells derived from mice vaccinated with HSP105-pulsed BM-DC were stimulated with HSP105-pulsed BM-DC three times in vitro. For the ELISPOT assay, these $CD8^+$ T cells were co-cultured with BM-DC prepulsed with HSP105, MBP, or unpulsed BM-DC for 24 h. (D) $CD8^+$ T cells stimulated with HSP105-pulsed BM-DC or BM-DC alone (control) were examined for their CTL activity against B16-F10 cells treated with IFN-γ (10^3 U/ml) using 6 h ^{51}Cr-release assay. The results were analyzed using the mean values of a triplicate or a quadruplicate assay. The data shown in A–D are each representative of three independent experiments with similar results.

tumor by stimulating T cells with tumor-specific peptides bound to HSPs. Subjeck and co-workers [27,28] reported that tumor-derived HSP110–peptide complexes also stimulated tumor immunity as other HSP families did in mice. Despite studies establishing a chaperoning effect of HSPs, one impediment to the full-fledged acceptance of HSPs as peptide-transporting vehicles is the lack of mass spectrometric data directly identifying HSP-associated peptides [29]. Stress-inducible proteins can be recognized by natural killer cells and CTLs as whole antigens expressed on the surface of stressed cells in humans [30]. Proteins dramatically upregulated or modified under stressful conditions should lead to increased presentation as do peptides presented by HLA class I molecules. About 25 HSP-derived peptides bound by HLA class I molecules have been identified through mass spectrometry [30]. Cancer patients have been reported to possess CTLs specific to HSP60-derived peptide [31], while HLA-A*0201-restricted HSP70-derived CTL epitopes have been identified in both an HLA-A*0201 transgenic mouse model and in humans [32]. In this study, although we did not identify HSP105-derived epitope peptides for $CD4^+$ T cells and $CD8^+$ T cells, we did prove that HSP105 itself could induce both $CD4^+$ Th-cells and $CD8^+$ CTLs specific to HSP105 as a cancer antigen. Contrary to our findings, however, Subjeck and co-workers [28] reported that HSP110 immunization did not elicit anti-tumor immunity. This discrepancy could be attributed to the difference in the methods of immunization.

It has been reported that HSPs can induce the maturation and activation of DCs as determined by upregulation of MHC class II and CD86 molecules, secretion of the IL-12 and TNFα [14,15]. However, HSP105-pulsed BM-DCs did not show any changes in comparison to the untreated BM-DC, thus suggesting that HSP105 did not induce DC maturation and activation. It is unlikely that HSP105 brought tumor-derived peptides into the culture system, because the HSP105 used in this study was the recombinant protein produced in *E. coli*. Furthermore, we recently identified HSP105-derived CTL epitopes restricted by HLA-A*0201 or -A*2402 using HLA

transgenic mouse model (unpublished data). These results also supported that HSP105 served not as a mediator for maturation of DCs, but as a cancer antigen eliciting tumor immunity.

The results of the T cell depletion study showed that the depletion of either $CD4^+$ T cells or $CD8^+$ T cells abrogated the anti-tumor immune response induced by the HSP 105-pulsed BM-DC vaccine, and that both $CD4^+$ and $CD8^+$ T cells play crucial roles in the protective anti-tumor immunity. $CD8^+$ T cells are thought to serve as the dominant effector cell mediating tumor killing, in contrast, $CD4^+$ T cells are thought to have an indirect role in providing help to CTL as well as a direct role in tumor rejection [33]. It is interesting that B16-F10 tumor cells that lack MHC class I were killed in in vivo study. We suppose that $CD4^+$ T cells may have an important role in this case. Peptides derived from HSP105 bound by MHC class II on the surface of HSP105-pulsed BM-DCs activate $CD4^+$ T cells. The activated $CD4^+$ T cells can secrete IFN-γ upon stimulation with tumor local DCs presenting tumor-derived HSP105 peptides, which contribute not only to activation of $CD8^+$ T cells but also to restoration of MHC class I expression in B16-F10 cells. The activated HSP105 specific $CD8^+$ T cells can recognize the peptides derived from HSP105 in the context of MHC class I and kill the B16-F10 cells.

In the field of cancer immunotherapy, most enthusiasm has been directed toward the use of various cancer vaccines; peptide vaccines alone, peptide plus cytokines, vaccination either with recombinant virus or with naked DNA encoding tumor antigen, and peptide pulsed on DCs [34]. DCs represent the most potent antigen presenting cells and also play an important role in the induction of specific T cell response [35]. Peptides pulsed on DCs have been reported to be the most effective vaccine in comparison to DNA vaccine or peptide–adjuvant mixture [36]. In this study, 62.5% and 80.0% of the mice immunized with HSP105-pulsed BM-DC completely rejected B16-F10 cells and C26 (C20) cells, respectively. On the other hand, only 50.0% of the mice immunized with the *HSP105*-DNA vaccine rejected these tumor cells in our previous study [13]. Although a further comparative analysis of the vaccination properties of these two strategies is required, our results suggested that protein-pulsed DCs are a more powerful vaccine than the DNA vaccine.

In this study, we used BM-DCs pulsed with HSP105 but not with HSP105-derived peptide as a cancer vaccine. We think that protein-pulsed DCs thus have an advantage over peptide-pulsed DCs. DCs are the major cell type known for its capacity to cross-present antigens [37]. In this study, HSP105-sensitized $CD8^+$ T cells responded to HSP105 in vitro by the stimulation of purified $CD8^+$ T cells with HSP105-pulsed DCs. This result strongly suggested that the HSP105-specific $CD8^+$ T cells were activated via the cross-presentation of HSP105 by BM-DCs. Although it became evident that gp96- and HSP70-chaperoned peptides can be presented to CTLs by DCs in the context of MHC class I molecules [38,39], we herein provide the first evidence that HSP itself can be cross-presented to CTLs by DCs. HSP105-pulsed DC can present peptides derived from exogenously added HSP105 in the context of not only MHC class II molecules on the surface of DCs to activate $CD4^+$ T cells, but also MHC class I molecules by cross-presentation to activate $CD8^+$ T cells. We herein showed the induction of specific $CD4^+$ T cells and $CD8^+$ T cells in vivo by stimulation with HSP105-pulsed DCs. The application of the peptide-pulsed DC as potential vaccine is limited to patients with the appropriate HLA alleles. To circumvent this limitation, we have used HSP105-pulsed DC to induce a HSP105 specific T cell response. HSP105-pulsed DCs offer the advantage of potentially presenting multiple immunogenic T cell epitopes without the need of prior knowledge of the individual patient's HLA type.

The mechanism of action of HSP105-pulsed BM-DCs injected intraperitoneally is still unclear. We think that DCs injected in the abdominal cavity might immigrate into mesenteric lymphatic vessels. Some DCs stay in mesenteric lymph nodes, others circulate in the blood via the thoracic duct and finally reach the spleen and bone marrow. Recent experimental evidence suggested that peripheral DCs migrate through the lymphatic vessels to the blood [40]. Although the present study showed that intraperitoneal injection of DCs induced an effective anti-tumor immunity in mice, comparison of effectiveness to other routes of immunization with DCs, such as intravenous, subcutaneous, and intranodal, remains to be investigated.

In conclusion, our results indicate that HSP105 itself is a tumor rejection antigen which may possibly be useful for cancer immunotherapy, and that HSP105-pulsed BM-DC vaccinations can prime HSP105-specific T cells in vivo, to prevent the subcutaneous growth of B16-F10 and C26 cancer cells expressing HSP105, without inducing autoimmune destruction. Our findings suggest that HSP105-pulsed BM-DC vaccination is a novel strategy for the prevention of cancer in patients treated surgically, who are at high risk for a recurrence of the cancer. Because of the overexpression of HSP105 in a variety of human tumors [12], clinical trial of immunotherapy targeted against HSP105 may well be applicable to various cancers.

Acknowledgments

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