Authors' contributions
ST designed, performed, and analyzed the experiments; coordinated collaborations; and wrote the manuscript. TO and YH performed and analyzed experiments. TY, HK, SS, and HM assisted in experimental design. KT, JAH, TK, CC, AN, and HS helped to analyze the data. TS contributed analysis of data. YN conceived of the project, designed the experiments, and analyzed the data. All authors read and approved the final manuscript.

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Sema4A inhibits the therapeutic effect of IFN-β in EAE

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ABSTRACT

Approximately one-third of patients with multiple sclerosis (MS) respond poorly to interferon-beta (IFN-β) therapy. Serum Sema4A is increased in MS patients, and those who have high Sema4A do not respond to IFN-β therapy. In this study, we investigated whether recombinant Sema4A abrogates the efficacy of IFN-β in mice with experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Administration of Sema4A concurrently with IFN-β diminished the efficacy of IFN-β in EAE. These effects of Sema4A were attributed to promote Th1 and Th17 differentiation and to increase adhesive activation of T cells to endothelial cells, even in the presence of IFN-β.

1. Introduction

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that causes neurological disability in young adults (Noseworthy et al., 2000). Although several disease-modifying drugs (DMDs) have been developed (Ransohoff, 2007; Pelletier and Hafler, 2012; Sempere et al., 2013), interferon-beta (IFN-β) remains one of the most prescribed treatments for relapsing-remitting multiple sclerosis (RRMS). IFN-β therapy reduces overall relapse rates by approximately 30% and improves prognosis (Rudick and Goelz, 2011).

The mechanisms of action of IFN-β have been studied utilizing experimental autoimmune encephalomyelitis (EAE), an animal model of MS, along with assessment of clinical responses to IFN-β therapy in humans. The mechanisms of action of IFN-β involve multiple immunoregulatory functions, including blocking the trafficking of lymphocytes to the CNS (Floris et al., 2002), reducing expression of major histocompatibility class II molecules (Jiang et al., 1995), attenuating T cell proliferation and altering the cytokine milieu from pro-inflammatory to anti-inflammatory (McRae et al., 1998; Kozovska et al., 1999; Ramgolam et al., 2009).

Although IFN-β therapy is safe and generally well tolerated, a major problem with this therapy is that approximately one-third of patients with MS do not respond to the therapy (Rio et al., 2006). Because early initiation of appropriate treatments is necessary for a better prognosis (Kappos et al., 2007), distinguishing responders from non-responders prior to the initiation of treatment is critical. Although no established biomarkers exist that predict responsiveness to IFN-β, Th17-skewing are suggested to be related to IFN-β resistance (Lee et al., 2011; Balasa et al., 2013). We previously reported that high levels of serum Sema4A are correlated with nonresponsiveness to IFN-β therapy (Nakatsuji et al., 2012). However, the underlying mechanisms of how Sema4A affects the response to IFN-β remain unknown.

Sema4A is a membrane-type class IV semaphorin that we originally identified using a dendritic cell cDNA library (Kumanogoh et al., 2002). Although semaphorins were originally identified as axon guidance molecules that act during neural development, Sema4A also plays a crucial role in the immune and vascular systems (Kumanogoh et al., 2002, 2005; Toyofuku et al., 2007). In the immune system, Sema4A activates Th cells and promotes differentiation of Th1 and Th17 cells, whereas it is involved in angiogenesis and migration of endothelial cells by modulating vascular endothelial growth factor signaling in the vascular system (Toyofuku et al., 2007; Meda et al., 2012). Consistent with its important role in Th cells, Sema4A has been implicated in EAE. Treatment with anti-Sema4A antibodies inhibits antigen-specific T cell generation and ameliorates EAE (Kumanogoh et al., 2002). In addition, Sema4A is

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increased in the sera of patients with MS (Nakatsuji et al., 2012). These data together suggest that Sema4A plays critical roles in both MS and EAE.

In this study, we investigated whether recombinant Sema4A abrogates the efficacy of IFN-β in the EAE model of MS and analyzed the underlying mechanisms.

2. Materials and methods

2.1. Animals & reagents

Wild-type C57BL/6 female mice were purchased from Oriental Yeast Corp. (Tokyo, Japan) and were maintained in a specific pathogen-free environment. Experimental procedures were approved by the Animal Care and Use Committee of Osaka University Graduate School of Medicine (Permit Number: 20-084-6). All possible efforts were made to minimize animal suffering and limit the number of animals used. Recombinant murine IFN-β (PBL Biomedical Laboratories, Piscataway, NJ) and recombinant human IFN-β 1b (Bayer, Osaka, Japan) were used. Sema4A-Fc protein was made as previously described (Kumanogoh et al., 2002). Briefly, we generated recombinant soluble mouse Sema4A protein comprising the putative extracellular region fused to the human immunoglobulin 1 (IgG1) Fc fragment.

2.2. MOG-specific T cell response and cytokine assay

Eight to 11 weeks old C57BL/6 female mice were immunized with 100 μg myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide (MEVGWYRSPFSPVVHLYRNGK) emulsified in complete Freund's adjuvant (CFA) containing 400 μg of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, MI, USA). For the recall assay, we collected the draining lymph nodes from mice 6 days after immunization. $CD4^+$ T cells were positively selected from the draining lymph nodes using an auto MACS cell purification system (Merck Millipore, Darmstadt, Germany), and 1×10^5 cells were restimulated for 72 hours with various concentrations of the MOG_{35-55} peptide in the presence of irradiated (3,000 rad) splenocytes (1×10^6 cells). Cytokines in the supernatant were assayed using mouse IFN-γ, IL-17A, IL-4 and IL-10 ELISA kits (R&D Systems, Minneapolis, MN, USA).

2.3. Induction of EAE and treatments

EAE was induced using a modification of our previously reported method (Takata et al., 2011). In brief, 8 to 11 weeks old C57BL/6 female mice were subcutaneously injected with 100 μg MOG_{35-55} emulsified in CFA supplemented with intraperitoneal injections of 200 ng pertussis toxin (List Laboratories, Campbell, CA, USA) on days 0 and 2.

For IFN-β treatment, IFN-β 1b (10,000 U) or phosphate buffered saline (PBS) was intraperitoneally injected to immunized mice every other day from days 0 to 10 after immunization. In addition, Sema4A-Fc (20 μg) or control human IgG (20 μg) was intravenously injected on days 0, 1, 3, 5, 7, 9 and 10 after immunization. All mice were monitored daily for clinical signs and were scored as follows using a scale of 0–5: 0, no overt signs of disease; 1, limp tail; 2, hind limb paralysis; 3, complete hind limb paralysis; 4, complete forelimb paralysis; 5, moribund state or death.

2.4. Histological and immunohistochemical analyses

Mice were sacrificed 22 days after immunization followed by transcardiac perfusion with 4% paraformaldehyde in PBS. For hematoxylin and eosin (H&E) staining, spinal cords were embedded in paraffin after perfusion with 4% PFA and 4 μm sections were cut from paraffin embedded blocks. Semi-quantitative histological analysis of inflammatory cellular infiltration was performed according to the following scoring system as previously described (Okuda et al., 2002): 0, no inflammation; 1, cellular infiltrates only in the perivascular areas and meninges; 2, mild cellular infiltration in the parenchyma; 3, moderate cellular infiltration in the parenchyma; 4, severe cellular infiltration in the parenchyma. For immunohistochemistry, Spinal cords were isolated, postfixed in 4% paraformaldehyde in PBS overnight, and then transferred to 30% sucrose in PBS overnight. Subsequently, the spinal cords were embedded in optimal cutting temperature (OCT) compound and frozen in liquid nitrogen. Frozen sections were cut at a thickness of 10 μm. For immunohistochemistry, the sections were incubated overnight at 4 °C with primary antibodies, followed by appropriate secondary antibodies for 2 hours at room temperature. The antibodies used in this study were as follows: rabbit anti-glial fibrillary acidic protein (GFAP) (1:500; Dako, Carpinteria, CA, USA), rabbit anti-Iba1 (1:500; Wako, Osaka, Japan), FITC-conjugated anti-CD3 (1:200; BD Biosciences, Franklin Lakes, NJ, USA), anti-von Willebrand factor (vWF) (1:500; Merck Millipore), anti-intercellular adhesion molecule (ICAM)-1 (1:50; BD Biosciences) and anti-vascular cell adhesion molecule (VCAM)-1 (1:50; Merck Millipore) antibodies. Images were captured by either a LSM510 laser scanning microscope (Zeiss, Thornwood, NY, USA) or a BZ9000 microscope (Keyence, Osaka, Japan). For quantification of T cells, microglia and astrocytes, we analyzed the density of CD3-, Iba1- or GFAP-positive cells, respectively, in every third sample section of horizontal spinal cord Sections. A total of 3 sections per mouse were analyzed and cell densities were counted in 4 randomly selected fields per section. We analyzed the area of double positive lesions of ICAM-1and vWF, and VCAM-1 and vWF in 4 randomly selected fields per section with NIS-elements software (Nikon, Tokyo, Japan). A total of 3 sections per mouse were analyzed.

2.5. Flow cytometry analysis

Lymphocytes were isolated from mice 6 days after immunization with MOG_{35-55}. Cells were washed twice with staining buffer containing 2% fetal bovine serum (FBS) and 0.09% sodium azide at pH 7.4 (BD Biosciences). To decrease non-specific cell staining, Fc receptors were blocked by incubating cells for 15 minutes on ice with an optimal concentration of rat anti-mouse CD16/CD32 antibody (1:50; BD Biosciences) diluted in staining buffer. Cells were then stained for 30 minutes on ice in the dark in the presence of APC-Cy7-labeled anti-CD4 antibody (1:100; BD Biosciences), followed by incubation with the following biotinylated anti-integrin antibodies: anti-α4, anti-αL, anti-β1 and anti-β2 (1:100; BD Biosciences). Thereafter, cells were washed twice with staining buffer and stained with FITC-conjugated streptavidin for 30 minutes. After staining, cells were washed twice with staining buffer, resuspended in staining buffer and passed through a Becton Dickinson FACS CANTO-2 ™ flow cytometer with Diva ™ software.

2.6. bEnd.3 cell culture and treatment

bEnd.3 cells, a murine brain cerebrovascular endothelial cell line, were maintained in DMEM (Wako) supplemented with 10% FBS and 100 U/mL penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). bEnd.3 cells were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air. Cells were stimulated with human IgG (20 μg/ml), Sema4A-Fc (20 μg/ml), IFN-β (5 U/ml) + human IgG (20 μg/ml) or IFN-β (5 U/ml) + Sema4A-Fc (20 μg/ml) for 6 hours. After stimulation, mRNA was collected for RT-RCR.

2.7. Adhesion assay

The adhesion of $CD4^+$ T cells to bEnd.3 cells was examined under static conditions as previously described with minor modifications (De Clerck et al., 1994). Briefly, we collected the lymphocytes from mice immunized with MOG_{35-55} emulsified in CFA 6 days after immunization. Lymphocytes from MOG_{35-55}-immunized mice were restimulated with the MOG_{35-55} peptide (50 μg/ml) for 24 hours. After restimulation,

$CD4^+$ T cells were positively collected from lymphocytes using an auto MACS cell purification system. $CD4^+$ T cells were labeled with 0.5 mM 2′,7′-bis(carboxyethyl)-5,6 carboxyfluorescein acetomethylester (BCECF-AM) (DOJINDO, Tokyo, Japan) in RPMI-1640 medium containing 10% FBS at 37 °C for 45 minutes and subsequently washed with PBS. bEnd.3 cells in 96-well plates were incubated for 24 hours and then treated with IFN-β (5 U/ml), recombinant Sema4A-Fc (20 μg/ml) or both for further 24 hours. Then, bEnd.3 cells were further incubated with BCECF-AM-labeled $CD4^+$ T cells at 37 °C for 1 hour. Thereafter, nonadherent cells were removed by washing with PBS. Fluorescence intensity was measured by SH-9000Lab (Corona Electric, Ibaraki, Japan).

2.8. Quantitative RT-PCR

RNA was isolated from bEnd.3 cells or homogenized flash-frozen mouse spinal cords and purified using ISOGEN (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's recommendations. cDNA was generated from 100 ng each RNA sample with an oligo dT primer and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) according to the manufacturer's recommendations.

Real-time RT–PCR was performed using the thermal cycler TaqMan 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Combined primers and probes were purchased from Applied Biosystems: Actb (Mm00607939_s1), Vcam1 (Mm01320970_m1), Icam1 (Mm00516023_m1), Ifng (Mm01168134_m1) and Il17a (Mm00439618_m1). PCR reactions were performed in triplicate in 20 μl using the Taq Man Universal PCR Master Mix (Applied Biosystems) and 1 μl cDNA. RT-PCR was carried out at 95 °C (10 minutes), followed by 40 cycles of 94 °C (1 minute), 56 °C (1 minute) and 72 °C (2 minutes). The relative expression levels of each mRNA were calculated using the ΔΔCt method and normalized to β-actin. Results are expressed relative to the control group.

2.9. Statistical analysis

EAE data, quantitative RT-PCR results and ELISA data are presented as means ± SEM. Statistical significance was determined with one-way ANOVA. p-values less than or equal to 0.05 were considered statistically significant.

3. Results

3.1. Sema4A promotes Th1 and Th17 differentiation in the presence of IFN-β

Induction of myelin-reactive Th1 and Th17 cells is the first crucial step for the initiation and progression of EAE and MS (Ishizu et al., 2005; Goverman, 2009). IFN-β suppresses the differentiation of these cells (Guo et al., 2008; Durelli et al., 2009; Zhang et al., 2011). To investigate the association between Sema4A and IFN-β in Th1 and Th17 differentiation, we first immunized C57BL/6 mice with MOG_{35-55} and treated them with recombinant Sema4A-Fc in the presence of IFN-β. $CD4^+$ T cells were collected from draining lymph nodes by positive selection, and the cytokines produced from MOG-reactive $CD4^+$ T cells following restimulation with MOG_{35-55} were examined. IFN-β treatment alone suppressed IFN-γ and IL-17 production (Fig. 1A and B), as described previously (Guo et al., 2008; Zhang et al., 2011). Sema4A alone did not change the production of IFN-γ and IL-17. However, Sema4A in addition to IFN-β reversed the suppression of these cytokines by IFN-β alone, and increased the production to the level of mice receiving control IgG (Fig. 1A, B). IL-4 was not detectable under any conditions (data not shown). The level of IL-10 was not altered by IFN-β and/or Sema4A (Fig. 1C). These data suggest that Sema4A promoted Th1 and Th17 differentiation in the presence of IFN-β.

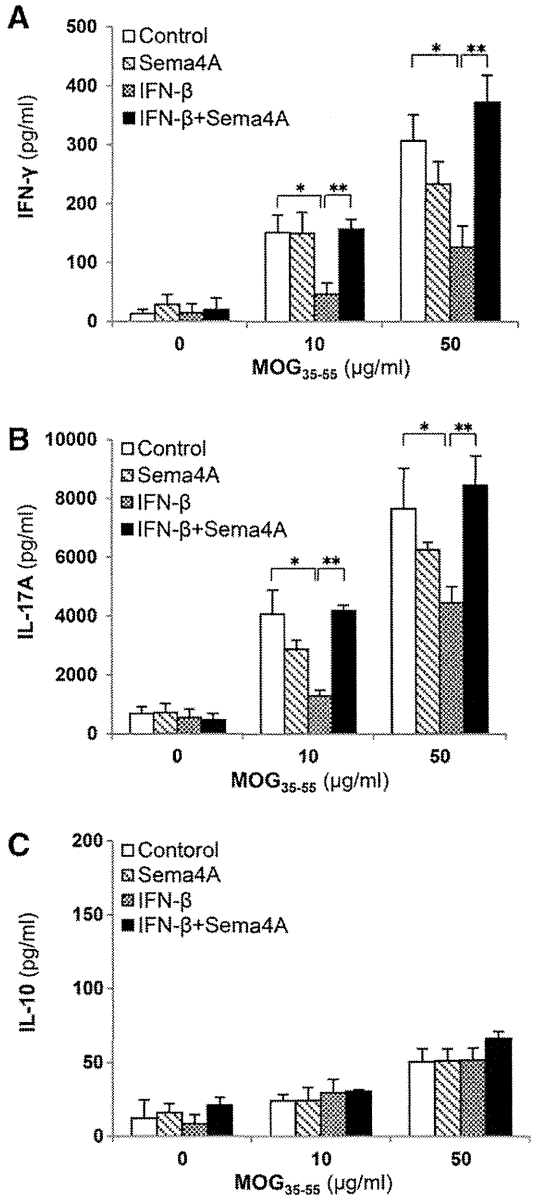

Fig. 1. Sema4A promotes Th1 and Th17 differentiation even in the presence of IFN-β. C57BL/6 mice were immunized with MOG_{35-55} and treated with PBS + human IgG (Control, n = 6), PBS + Sema4A-Fc (Sema4A, n = 6), IFN-β + human IgG (IFN-β, n = 6) or IFN-β + Sema4A-Fc (IFN-β + Sema4A, n = 6). Six days after immunization, $CD4^+$ T cells from the draining lymph nodes were restimulated for 72 hours with various concentrations of MOG_{35-55} in the presence of irradiated splenocytes from C57BL/6 mice. IFN-γ, IL-17 and IL-10 in the culture supernatants were assayed by ELISA. Data are the mean ± SEM of two independent experiments. $^*p \leq 0.05$; $^{**}p \leq 0.01$.

3.2. Sema4A promotes T cell adhesion to endothelial cells

After differentiation of encephalitogenic T cells, their adhesion to the endothelium is required for entry into the CNS and development of EAE and MS (Palmer, 2013). Very late antigen-4 (VLA-4, α4β1 integrin) and leukocyte function-associated antigen-1 (LFA-1, αLβ2 integrin) are needed for T cell tethering to the endothelium (Archelos et al., 1999). We examined whether the expression of VLA-4 and LFA-1 on $CD4^+$ T cells is increased by Sema4A. Recombinant Sema4A-Fc or IFN-β was administered to MOG_{35-55}-immunized C57BL/6 mice, and $CD4^+$ T cells were collected from draining lymph nodes to analyze the expression of VLA-4 and LFA-1. The expression levels of these integrins were not affected by Sema4A, IFN-β or a combination of these (Fig. 2A).

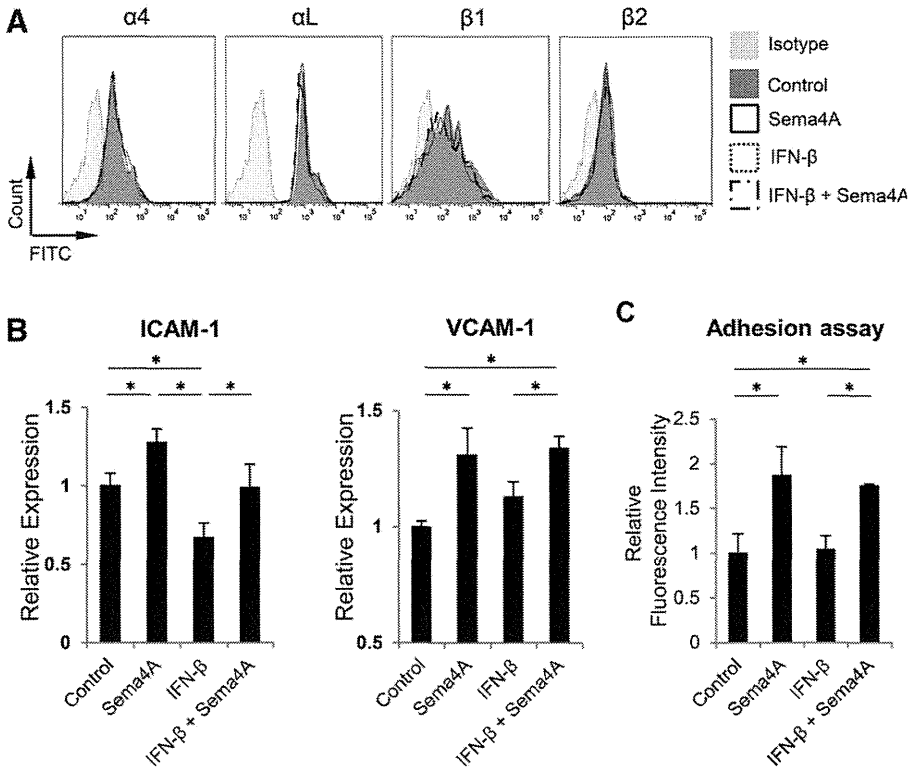

Fig. 2. Sema4A promotes T cell adhesion to endothelial cells. $CD4^+$ T cells were isolated from draining lymph nodes of MOG_{35-55}-immunized mice, which were treated with human IgG (Control), Sema4A-Fc (Sema4A), IFN-β + human IgG (IFN-β) or IFN-β + Sema4A-Fc (IFN-β + Sema4A) (n = 4 per each group). (A) α4, αL, β1 and β2 integrins on $CD4^+$ T cells were analyzed by flow cytometry. Sema4A did not affect the expression of LFA-1 or VLA-4. Data are representative of two independent experiments. (B) Relative mRNA expression of ICAM-1 and VCAM-1 in bEnd.3 cells. bEnd.3 cells were incubated for 1 day with human IgG (Control, n = 9), Sema4A–Fc (Sema4A, n = 9), IFN-β + human IgG (IFN-β, n = 7) or IFN-β + Sema4A-Fc (IFN-β + Sema4A, n = 7). Data are the mean ± SEM of four independent experiments. $^*p \leq 0.05$ (C) T cell adhesion to endothelial cells. bEnd.3 cells were stimulated with human IgG (Control, n = 3), Sema4A-Fc (Sema4A, n = 3), IFN-β + human IgG (IFN-β, n = 3) or IFN-β + Sema4A-Fc (IFN-β + Sema4A, n = 3). $CD4^+$ T cells were isolated from the spleens of MOG_{35-55}-immunized mice (n = 3). $CD4^+$ T cells were labeled with BCECF-AM and incubated on a bEnd.3 cell layer for 1 h. Then, nonadherent cells were removed, and the fluorescence intensity was measured. Sema4A promoted T cell adhesion to endothelial cells regardless of IFN-β treatment. Data are the mean ± SEM from a representative of three independent experiments. $^*p \leq 0.05$.

Next, we examined whether VCAM-1 and ICAM-1, ligands for VLA-4 and LFA-1, respectively that are expressed on endothelial cells, are affected by Sema4A with or without IFN-β. bEnd.3 cells were incubated with recombinant Sema4A-Fc in the presence or absence of IFN-β, and then the expression of mRNA for VCAM-1 and ICAM-1 was analyzed using quantitative RT-PCR. We found that Sema4A alone increased the expression level of ICAM-1 and VCAM-1 in endothelial cells (Fig. 2B). The enhanced expression of ICAM-1 and VCAM-1 by Sema4A was observed even in the presence of IFN-β. Consistent with RT-PCR analysis, immunohistochemical analysis also showed Sema4A enhanced the expression of ICAM-1 and VCAM-1 in bEnd.3 compared to those stimulated control IgG (Supplementary Fig. 1A and B). Furthermore, we performed an adhesion assay using splenic $CD4^+$ T cells and bEnd.3 cells in the presence of recombinant Sema4A-Fc and IFN-β. $CD4^+$ T cells were incubated with a cell layer of bEnd.3 cells that had been treated with Sema4A. Adhesion of T cells to the bEnd.3 cell layer was significantly increased compared to cells not treated with Sema4A (Fig. 2C). These results suggest that Sema4A promotes T cell adhesion to endothelial cells by increasing ICAM-1 and VCAM-1, even in the presence of IFN-β.

3.3. IFN-β is not effective in mice with EAE that received recombinant Sema4A-Fc

To determine whether Sema4A inhibits the beneficial effect of IFN-β in vivo, recombinant Sema4A-Fc was administered to mice with EAE that were also treated with IFN-β. IFN-β in the absence of Sema4A delayed the onset and attenuated the disease severity of EAE, as previously reported (Inoue et al., 2012). Although Sema4A alone did not affect the severity, administration of Sema4A concurrently with IFN-β resulted in exacerbation of EAE to a level similar to or worse than that in the control group (Fig. 3A).

Consistent with the clinical score, histological analysis with H&E staining of the spinal cord showed that mice receiving IFN-β exhibited reduced cellular infiltration, whereas Sema4A administration in addition to IFN-β increased cellular invasion compared to mice treated with IFN-β alone (Fig. 3B and C).

To further investigate the mechanism by which Sema4A abrogates the beneficial effect of IFN-β in EAE, spinal cord sections were stained with anti-CD3, -Iba1 and -GFAP antibodies to assess T cells, microglia and astrocytes, respectively. T cell infiltration and the activation of microglia and astrocytes were remarkably reduced in mice treated with IFN-β compared to control mice (Fig. 4A and B). Decreased cellular infiltration by IFN-β treatment was accompanied by a reduction in the mRNA expression of IFN-γ and IL-17 in the spinal cord (Fig. 4C). These results suggest that IFN-β ameliorates EAE by inhibiting Th1 and Th17 pathology. Administration of recombinant Sema4A alone did not significantly affect the mRNA expression of IFN-γ and IL-17, and no apparent differences in T cell infiltration or glial activation were observed compared to the control group. In contrast, when Sema4A was administered in addition to IFN-β, T cell infiltration and glial activation were apparently increased compared to mice receiving IFN-β alone (Fig. 4A and B). The decreased expression of IFN-γ and IL-17 mRNA by IFN-β was abrogated by additional administration of Sema4A (Fig. 4C). Consistent with mRNA levels of these cytokines, IFN-β alone decreased IFN-γ-positive cells and IL-17-positive cells in spinal cords, whereas Sema4A

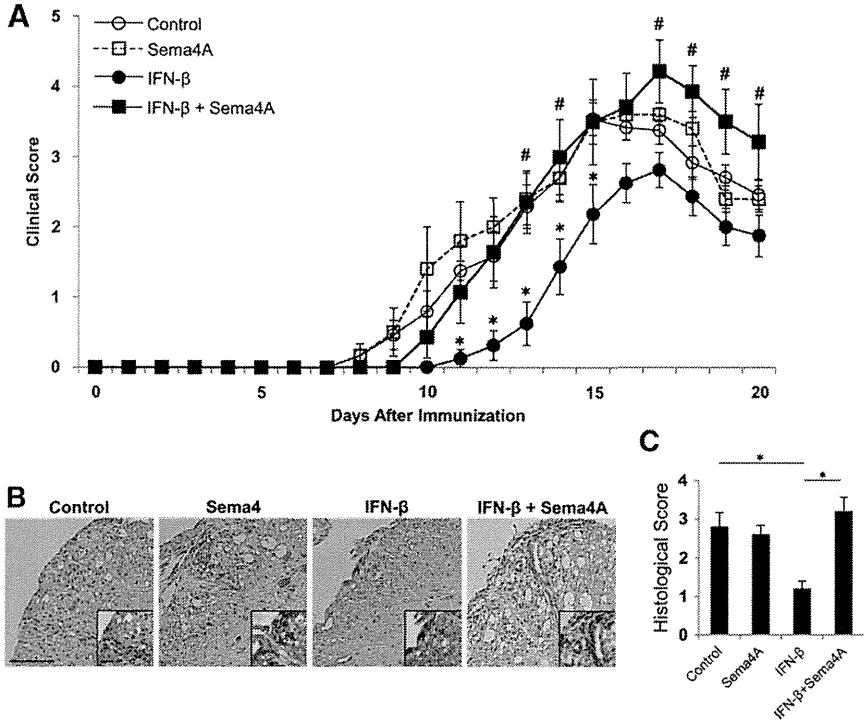

Fig. 3. Sema4A inhibits the efficacy of IFN-β in mice with EAE. (A) The mean clinical scores of mice with EAE are shown. Immunized mice were treated with PBS + human IgG (Control; open circles, n = 10), PBS + Sema4A-Fc (Sema4A; open squares, n = 8), IFN-β + human IgG (IFN-β; filled circles, n = 8) or IFN-β + Sema4A-Fc (IFN-β + Sema4A; filled squares, n = 8). Sema4A exacerbated the clinical score to the level of the control group, even in the presence of IFN-β. Data represent the mean score ± SEM of two independent experiments. $^{*}p \leq 0.05$ for IFN-β versus Control; $^{\#}p \leq 0.05$ for IFN-β + Sema4A versus IFN-β. Representative images of H&E staining of spinal cords on day 22 post-immunization (B) and the histological score (C) are shown. Sema4A promoted cellular infiltration in the presence of IFN-β. The insets in (B) show higher magnification of the spinal cords. Scale bars: 400 μm. Each bar in (C) indicates the mean pathological score ± SEM of three mice from each group. $^{*}p \leq 0.05$.

in addition to IFN-β increased infiltration of these cells to the level of control group (Supplementary Fig. 2A and B).

Because our in vitro data suggested that Sema4A promoted T cell adhesion to endothelial cells by increasing ICAM-1 and VCAM-1 expression, we next examined the expression of these adhesion molecules in the spinal cord of mice with EAE. The expression of VCAM-1 mRNA was significantly increased in mice treated with IFN-β plus Sema4A compared to mice treated with IFN-β alone. ICAM-1 expression also tended to increase following treatment with IFN-β plus Sema4A compared to mice treated with IFN-β alone (Fig. 4C), though the difference was not significant. Immunohistochemical analysis revealed that ICAM-1 and VCAM-1 were expressed on vWF-positive endothelial cells. Expression of ICAM-1 and VCAM-1 was increased in mice treated with IFN-β plus Sema4A compared to mice treated with IFN-β alone (Fig. 4D and E).

These data together suggest that Sema4A enhances not only Th1 and Th17 differentiation but also T cell adhesion to endothelial cells in the presence of IFN-β.

4. Discussion

In the current study, we investigated the implications of Sema4A on the efficacy of IFN-β by administering recombinant Sema4A-Fc and IFN-β to mice with EAE.

IFN-β confers its beneficial effect in MS and EAE through pleiotropic mechanisms of action. Among these, suppression of encephalitogenic T cells is one of the most important mechanisms. Reduction of T cell activation and proliferation, and induction of a shift toward anti-inflammatory cytokines have been shown to contribute to the efficacy of IFN-β (Kieseier, 2011). Concerning effector Th cell differentiation, IFN-β inhibits Th1 and Th17 differentiation (McRae et al., 1998; Durelli et al., 2009; Ramgolam et al., 2009). In contrast to the effects of IFN-β, Sema4A activates T cells and promotes Th1 and Th17 differentiation. Therefore, Sema4A may antagonize the suppressive effects of IFN-β on encephalitogenic T cells. Indeed, deterioration of EAE by Sema4A in the presence of IFN-β was accompanied by an increase in IFN-γ and IL-17 production by MOG-reactive Th cells as shown in Fig. 1.

We previously reported that the condition of MS patients with high Sema4A tends to deteriorate after IFN-β therapy. In the present study, recombinant Sema4A alone did not affect Th1 and Th17 reactions. However, Sema4A promoted the differentiation of these cells only when it was administered in combination with IFN-β. These data suggest that IFN-β may promote encephalitogenic T cell activation signaling downstream of Sema4A. IFN-β has not only anti-inflammatory but also pro-inflammatory effects including enhancing Th1 differentiation (Nagai et al., 2003) and increasing IL-6, an inflammatory cytokine that is required for development of Th17 cells (Ivanov et al., 2006; Nakatsuji et al., 2006; Kimura et al., 2007) under certain conditions. Indeed, patients with neuromyelitis optica, who are thought to have more IL-6 involvement than MS patients (Chihara et al., 2011), have been shown to deteriorate after receiving IFN-β therapy (Shimizu et al., 2010). Therefore, Sema4A may enhance IFN-β-induced pro-inflammatory reactions followed by increased pathogenicity of encephalitogenic T cells.

Th17 pathology has been implicated in the IFN-β resistance in MS (Axtell et al., 2010; Balasa et al., 2013). Axtell et al. reported that IFN-β exacerbates EAE induced by adoptive transfer of Th17 cells (Axtell et al., 2010) and suggested that IFN-β enhances Th17-mediated inflammation in the effector phase. Because MS patients with high Sema4A exhibit skewing towards Th17 condition, Sema4A and Th17 are thought to be closely linked in the context of IFN-β resistance. Indeed, we demonstrated that Sema4A promoted Th17 differentiation in the presence of IFN-β treatment. Therefore, exacerbation of EAE in the presence of

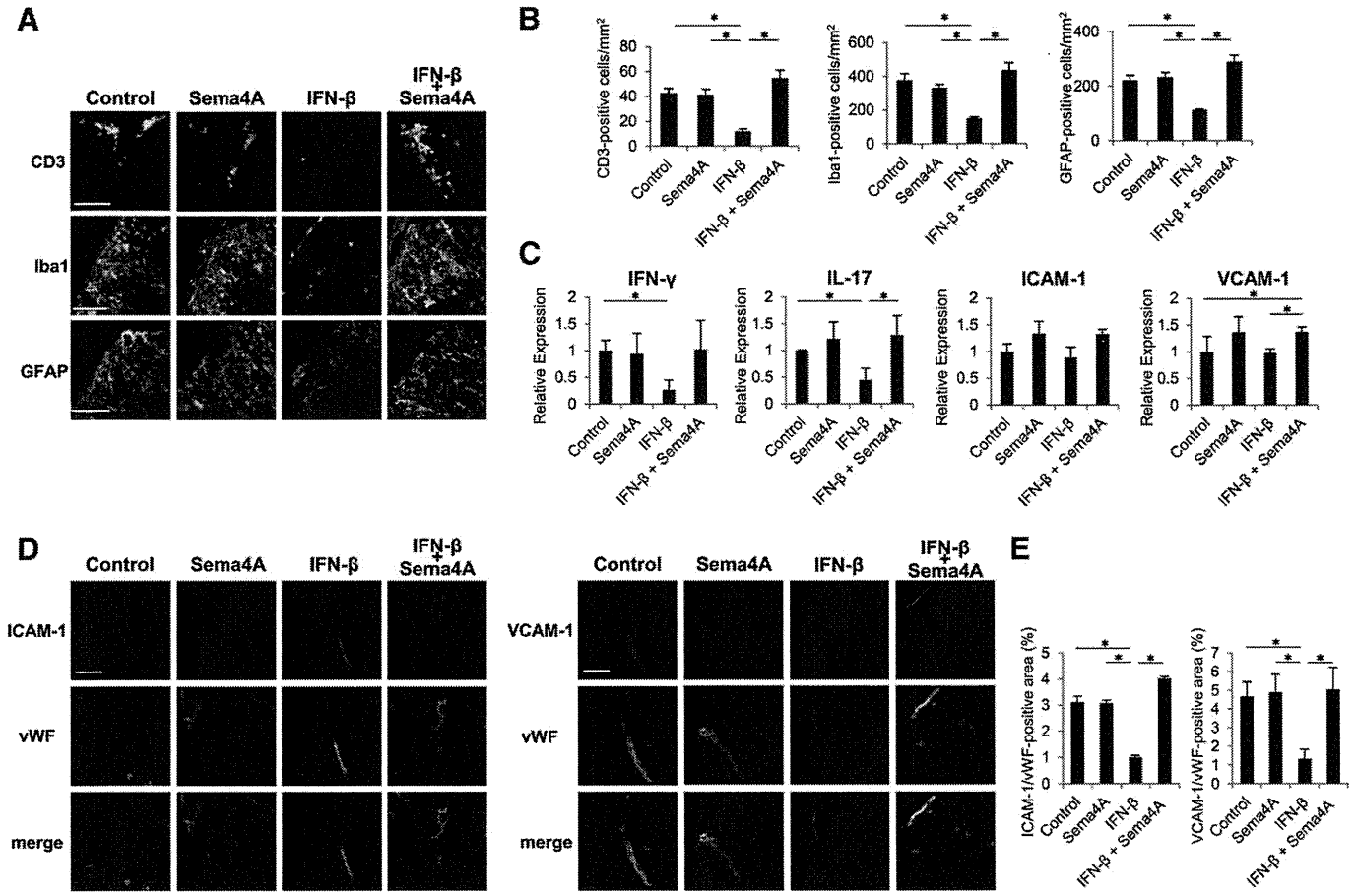

Fig. 4. Sema4A promotes T cell infiltration into the CNS and increases the expression of ICAM-1 and VCAM-1 in the CNS of mice with EAE. (A) Immunostaining with anti-CD3, -Iba1 and -GFAP antibodies in spinal cords of mice with EAE is shown. Mice with EAE were treated with PBS + human IgG (Control), PBS + Sema4A-Fc (Sema4A), IFN-β + human IgG (IFN-β) or IFN-β + Sema4A-Fc (IFN-β + Sema4A). Spinal cords were collected 22 days after immunization. Scale bars: anti-CD3; 100 μm, anti-Iba1; 50 μm, anti-GFAP; 50 μm. (B) The numbers of CD3-, Iba1- and GFAP-positive cells of spinal cord in each group are shown. Data presented in (A) and (B) is a representative of two animals. $^{*}p \leq 0.05$ (C) Expression of mRNA for IFN-γ, IL-17, ICAM-1 and VCAM-1 relative to that of mice with EAE treated with PBS + human IgG is shown (n = 4–5 per group). RNA was extracted from spinal cords of mice with EAE 21 days after immunization. Sema4A counteracted the inhibition of IFN-γ and IL-17 expression by IFN-β and promoted the expression of ICAM-1 and VCAM-1. Data are the mean ± SEM from a representative of two independent experiments. Double immunofluorescent staining with anti-ICAM-1/vWF and anti-VCAM-1/vWF in the spinal cord of mice with EAE (D) and quantitative analysis of immunohistochemical staining (E) are shown. Sema4A increased the expression of ICAM-1 and VCAM-1. Data presented in (D) and (E) is a representative of two animals. Scale bars: 50 μm. $^{*}p \leq 0.05$.

both Sema4A and IFN-β treatment may be partially due to enhanced Th17 differentiation by Sema4A.

In addition to the effect on encephalitogenic T cells, IFN-β has a profound effect on T cell migration into the CNS (Palmer, 2013). Of note, we found that Sema4A promoted T cell adhesion to endothelial cells by increasing the expression of ICAM-1 and VCAM-1 on endothelial cells. In this context, Sema4A may antagonize the efficacy of IFN-β, which limits lymphocyte migration across the blood–brain barrier. However, because Sema4A alone did not exacerbate the severity of EAE compared to controls, enhancement of T cell adhesion by Sema4A alone may not be sufficient to induce deterioration of EAE and MS. Sema4A-induced T cell adhesion may contribute to the pathogenesis of EAE and MS when combined with enhanced pathogenicity of encephalitogenic T cells.

Consistent with our previous study of patients with MS, Sema4A inhibited the therapeutic effect of IFN-β in mice with EAE. This abrogation was accompanied by an increase in antigen-specific Th1 and Th17 cell differentiation and T cell adhesion to endothelial cells. These results not only suggest a close link between Sema4A and IFN-β resistance but also support the notion that MS patients with high Sema4A will be non-responders to IFN-β therapy. Our findings imply that DMDs other than IFN-β may be appropriate for treatment of patients with MS who have high levels of Sema4A. Further investigation is required to select appropriate DMDs for MS patients with high Sema4A.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2013.12.014.

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ORIGINAL

Effects of exenatide on metabolic parameters/control in obese Japanese patients with type 2 diabetes

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Abstract. The effects of exenatide on glycemic control, lipid metabolism, blood pressure, and gastrointestinal symptoms were investigated in obese Japanese patients with type 2 diabetes mellitus. Twenty-six outpatients were enrolled and administered 5 μg of exenatide twice daily. If there was insufficient weight loss and/or insufficient improvement in glycemic control, the dose was increased to 10 μg twice daily. Follow-up was continued until the 12th week of administration. Hemoglobin A1c, glycoalbumin, fasting plasma glucose, body weight, fasting serum C-peptide, serum lipids, blood pressure, and pulse rate were measured before and after the observation period. In the initial phase of exenatide therapy, each patient received a diary to record gastrointestinal symptoms. During treatment with exenatide, hemoglobin A1c decreased significantly and serum C-peptide increased significantly. Body weight, low-density lipoprotein cholesterol, and systolic blood pressure decreased significantly. Nausea was the most frequent gastrointestinal symptom and occurred in 16 patients. Its onset was noted at a mean of 1.7 h after injection, the mean duration was 1.1 h, and it continued for a mean of 9.3 days after the initiation of administration. Patients with nausea showed a significant decrease in hemoglobin A1c, glycoalbumin, or body weight compared with those without nausea. These findings suggest that a more marked improvement in metabolic parameters by exenatide can be partly dependent on the manifestation of gastrointestinal symptoms.

Key words: Type 2 diabetes mellitus, Obesity, Exenatide, Incretin

EXENATIDE is a human glucagon-like peptide-1 (GLP-1) receptor agonist produced by solid-phase peptide synthesis that has the same amino acid sequence as exendin-4, which was isolated from the saliva of the Gila monster (Heloderma suspectum). Similar to endogenous active GLP-1, exenatide specifically binds to GLP-1 receptors (seven-transmembrane, G protein-coupled receptors) and activates adenylate cyclase to increase the intracellular concentration of cyclic AMP in β-cells, leading to a decrease in plasma glucose levels by promoting glucose-dependent insulin secretion. In addition to the suppression of increased glucagon secretion, exenatide promotes weight loss caused by delayed gastric emptying and/or suppression of food intake through the satiety effect [1].

The most frequent gastrointestinal symptoms caused by exenatide are nausea, vomiting, diarrhea, and constipation, which may be related to delayed gastric emptying, suppressed food intake and enhanced colonic motility, although the detailed mechanisms have yet to be clarified.

Few reports have been published on the effects of exenatide on glycemic control and gastrointestinal symptoms in obese Japanese patients with type 2 diabetes. Therefore, we investigated the effects of exenatide on metabolic parameters such as plasma glucose, lipids, blood pressure, and body weight and evaluated the associated gastrointestinal symptoms in Japanese obese patients with type 2 diabetes.

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Patients and Methods

Patients

From August 2011 to September 2012, 26 obese

outpatients with type 2 diabetes mellitus were enrolled in this study (obesity is defined by a body mass index (BMI) of ≥25kg/m^2 in Japan). All patients had inadequate glycemic control after more than 6 months of treatment with oral hypoglycemic agents and insulin in addition to diet and exercise. Other enrollment criteria for this study were as follows: hemoglobin A1c (HbA1c; NGSP) ≥7.0%; BMI ≥25 kg/m^2; and fasting serum C-peptide reactivity (CPR) ≥1.0 ng/mL.

Exclusion criteria included severe hepatic/renal disorders, severe infection, recent or scheduled surgery, severe trauma, and other factors deemed inappropriate by the attending physician.

Previous medications included insulin in 9 patients (38%), dipeptidyl peptidase 4 (DPP-4) inhibitor in 10 (42%), sulfonylureas in 11 (46%), metformin in 9 (38%), α-glucosidase inhibitor (α-GI) in 5 (21%), thiazolidine in 3 (13%), and diet and exercise alone in 3 (13%).

Study design

The effects of additional treatment with exenatide were assessed in week 12.

Various metabolic parameters such as body weight, fasting plasma glucose, HbA1c (NGSP), glycoalbumin (GA), serum CPR, lipids [low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides], and blood pressure (systolic and diastolic) were measured before treatment (week 0) and in week 12 of exenatide treatment.

For initial treatment, 5 μg of exenatide was subcutaneously administered twice a day (30 min before breakfast and 30 min before dinner). When weight reduction or improvement in HbAlc was not observed by week 4 in comparison with week 0, the dosage was increased to 10 μg twice daily until week 12.

Oral hypoglycemic agents used prior to exenatide treatment were continued at the same dose; however, insulin, DPP-4 inhibitor and a-glucosidase inhibitor were discontinued. Other drugs were not added or changed during the study period. At the start of exenatide administration, a diary was given to each patient so that a daily record of the presence/absence and details of any gastrointestinal symptoms could be maintained.

Initial analysis was conducted for all patients, who were then divided into two groups: a group in which nausea was the most frequent gastrointestinal symptom during the period from the start of exenatide administration until week 12 and a group in which nausea was not reported throughout the study period. The relationship between each parameter and gastrointestinal symptoms was then assessed.

The aims of the study were explained to all patients and written informed consent was obtained. Moreover, this study was approved by the ethical committee of Hyogo College of Medicine and was conducted according to the Declaration of Helsinki.

Statistical analysis

StatView ver. 5.0 (SAS Institute Inc, Cary, NC, USA) was used for statistical analyses. The paired t-test or Wilcoxon signed-rank test was used for the comparison of parameters between Week 0 and Week 12, while the unpaired t-test or the Mann–Whitney test was used for comparison between the two groups. Statistical significance was set at $p < 0.05$. Simple correlations (Pearson) were used to assess the correlation of the number of days when nausea occurred, the total duration of nausea, and the time of occurrence after exenatide injection with the following parameters: HbAlc, GA, body weight, BMI, CPR, lipids (LDL cholesterol, HDL cholesterol, triglycerides), and blood pressure. Results are expressed as mean ± SD.

Results

Results for all patients (24 patients, excluding 2 dropouts)

The patient profiles are shown in Table 1. Of the 26 enrolled patients, 2 discontinued the study within 4 weeks of starting exenatide treatment because of severe gastrointestinal symptoms (nausea and vomiting). In the remaining 24 patients, HbAlc decreased significantly between week 0 and week 12 (9.3%–8.0%, $p = 0.004$) (Fig. 1a). GA also showed a significant change (23.4%–20.4%, p=0.033) (Fig. 1b). No significant change was found in fasting plasma glucose (166–164 mg/dL;) (Fig. 1c). However, a significant increase in fasting CPR was observed (2.18–2.89 ng/mL; $p = 0.0028$) (Fig. 1d). Weight loss was significant (75.2–71.4 kg; $p < 0.0001$) (Fig. 2a), with a consequent decline in BMI (28.8–27.4 kg/m^2; $p < 0.0001$) (Fig. 2b). Furthermore, a significant decrease in systolic blood pressure (136–130 mmHg; $p = 0.008$) (Fig. 2c) was observed, whereas diastolic blood pressure showed no significant change (79–78 mmHg;) (data not shown). There was a significant decrease in LDL cholesterol (121–110 mg/dL; $p = 0.023$) (Fig. 2d), whereas no significant change was observed in HDL cholesterol

Table 1 Patient profiles

	total (n = 24)	with nausea (n = 14)	without nausea (n = 10)	*p* value
Gender (male : female)	10 : 14	5 : 9	5 : 5	
Age (y)	57.3±13.2	59.7±11.8	54.0±14.3	n.s
Height (cm)	160.8±10.7	158.3±10.6	164.3±9.9	n.s
Body weight (kg)	75.2±13.9	72.2±12.9	79.3±14.2	n.s
Body mass index (kg/m^2)	28.9±2.9	28.7±2.9	29.2±2.9	n.s
Duration of DM (years)	9.5±7.9	8.3±7.6	11.9±8.0	n.s
HbA1c (NGSP) (%)	9.3±1.3	9.0±1.5	9.6±0.7	n.s
GA(%)	23.4±4.8	22.1±4.8	25.2±4.2	n.s
Fasting plasma glucose (mg/dL)	166±47	158±34	178±60	n.s
Fasting plasma CPR (ng/mL)	2.2±1.2	2.2±1.0	2.2±1.5	n.s
SBP (mmHg)	136±16	141±17	129±12	n.s
DBP (mmHg)	79±14	81±13	77±15	n.s
Pulse rate (bpm)	82±10	83±9	81±12	n.s
LDL-chol (mg/dL)	121±27	121±26	124±40	n.s
HDL-chol (mg/dL)	51±13	50±10	53±17	n.s
Triglyceride (mg/dL)	163±76	176±70	146±81	n.s
Daily dose of exenatide (10μg : 20μg)	17 : 7	12 : 2	5 : 5	

Data are expressed as mean ± SD.
n.s., not significant; GA, glycoalbumin; CPR, C-peptide reactivity; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-chol, low-density lipoprotein cholesterol; HDL-chol, high-density lipoprotein cholesterol

(51–49 mg/dL; data not shown) and triglycerides (163–167 mg/dL; data not shown).

Gastrointestinal symptoms

Among the confirmed gastrointestinal symptoms, nausea was the most common, occurring in 16 of the original 26 patients (62%) who received exenatide (Table 2). Vomiting occurred in 3 patients (12%) and diarrhea in 2 (8%).

In the patients who developed nausea, the mean time of onset after exenatide injection was 1.6 ± 1.2 h, while the mean duration of each episode was 1.1 ± 0.5 h. Nausea occurred for a mean of 9.3 ± 6.4 days from the initiation of administration.

There was no case in which gastrointestinal symptoms reappeared or newly appeared after the dose of exenatide was increased to 10 μg 4 weeks after the initiation of administration.

Correlation of gastrointestinal symptoms with metabolic parameters

Correlations of the number of days for which nausea occurred, the time of onset, and the duration of each episode with metabolic parameters such as HbAlc, GA, serum CPR, lipids, body weight, BMI, and blood pressure were investigated in all 24 patients. No significant correlations were observed. Then, the patients who received 12 weeks of exenatide administration were divided into two groups. One group of 14 patients experienced nausea, and the other group of 10 did not experience nausea throughout the study period. Comparison of these two groups revealed that the group with nausea showed a significant decrease in HbAlc (9.0%–7.5%, $p = 0.0167$) (Fig. 1a) and GA (22.1%–18.0%; $p = 0.0171$) (Fig. 1b) between week 0 and week 12. There was no significant change in fasting plasma glucose (158–146 mg/dL) (Fig. 1c). In the group without nausea, there was no significant change in HbAlc (9.6%–8.7%) (Fig. 1a) or GA (25.2%–23.7%) (Fig. 1b). Fasting plasma glucose increased from 178 mg/dL to 190 mg/dL, but the change was not significant ($p = 0.609$) (Fig. 1c).

The group with nausea showed no significant change in fasting serum CPR from week 0 to week 12 (Fig. 1d), whereas the group without nausea showed a significant increase (2.22–3.48 ng/mL; $p = 0.002$) (Fig. 1d).

Body weight decreased significantly (72.2–68.1 kg; $p = 0.0003$) (Fig. 2a) in the group with nausea, with a consequent decline in BMI (28.7–26.9 kg/m^2; $p = 0.0003$) (Fig. 2b). Significant weight loss was also observed in the group without nausea (79.3–76.2 kg; $p = 0.012$) (Fig. 2a), with a consequent decline in BMI (29.2–27.9 kg/m^2, $p = 0.015$) (Fig. 2b). The decrease was greater in patients with nausea than in those without.

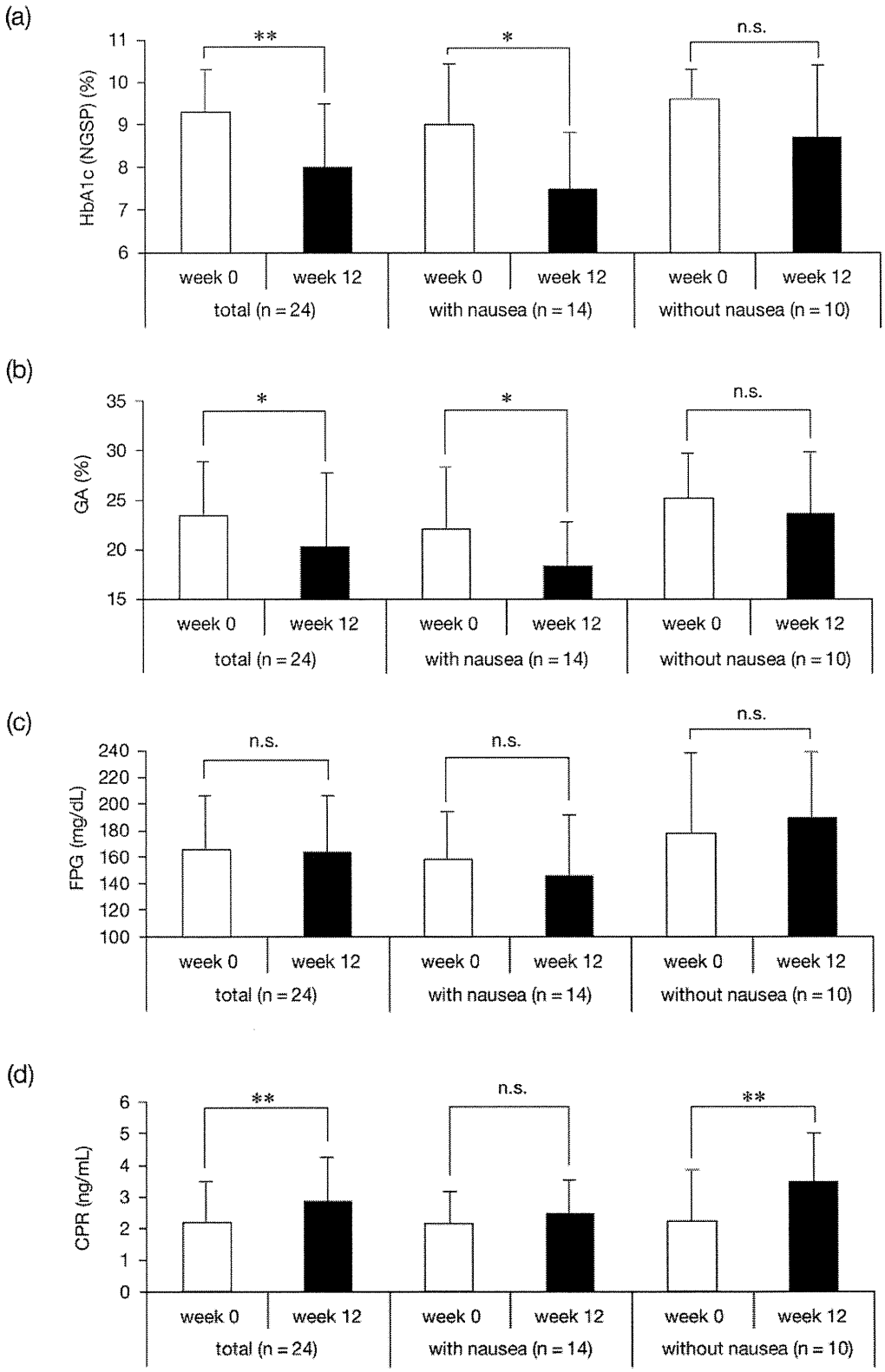

Fig. 1 Changes in HbA1c (a), GA (b), FPG (c), and fasting plasma CPR (d) with exenatide
*$p < 0.05$; **$p < 0.005$; n.s., not significant
GA, glycoalbumin; FPG, fasting plasma glucose; CPR, C-peptide reactivity

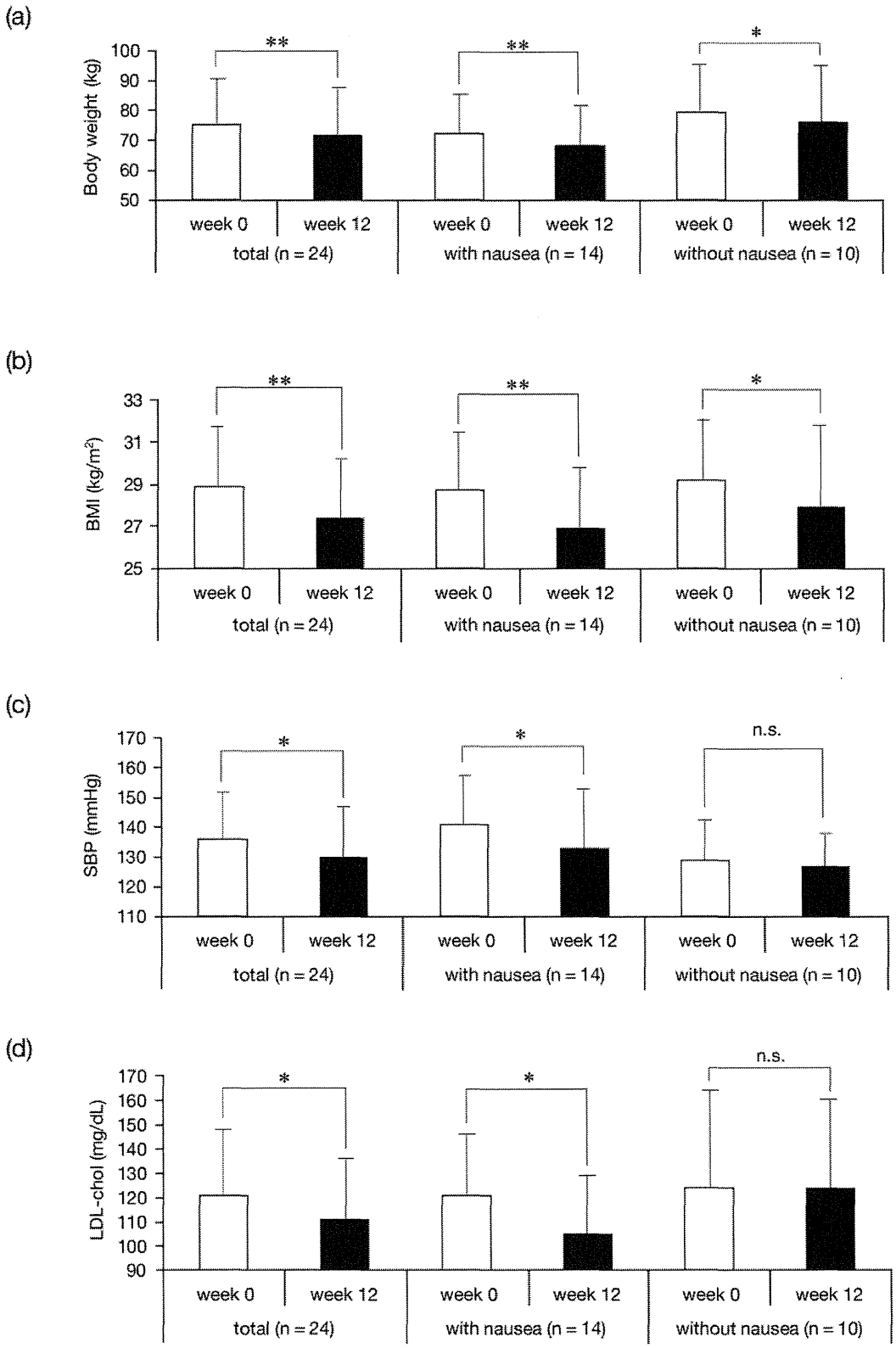

Fig. 2 Changes in body weight (a), BMI (b), SBP (c), and LDL cholestrol (d) with exenatide
*$p < 0.05$; **$p < 0.005$; n.s., not significant
BMI, body mass index; SBP, systolic blood pressure; LDL-chol, low-density lipoprotein cholesterol

Table 2 Gastrointestinal symptoms (n = 26)

Nausea (%)	16 (62)
Vomiting (%)	3 (12)
The time when nausea appears after injection (h)	1.6±1.2
The duration of nausea continued (h)	1.1±0.5
The days which nausea follows (day)	9.3±6.4

The group with nausea showed a significant decrease in systolic blood pressure between week 0 and week 12 (141–133 mmHg; $p = 0.0208$) (Fig. 2c), but no significant change was observed in diastolic blood pressure and pulse rate (data not shown). The group without nausea showed no significant changes in systolic blood pressure, diastolic blood pressure, and pulse rate.

The group with nausea showed a significant decrease in LDL cholesterol (was observed) (125–105 mg/dL; $p = 0.0133$) (Fig. 2d), but no significant change in HDL cholesterol and triglycerides (data not shown) between week 0 and 12. The group without nausea showed no significant change in LDL cholesterol (Fig. 2d), HDL cholesterol, and triglycerides (data not shown).

Effects of prior treatment with insulin and DPP-4 inhibitor on plasma glucose, CPR and body weight

HbA1c in 9 patients treated with insulin prior to the treatment with exenatide (insulin group) was 9.1 ± 1.2% at week 0 and 8.6 ± 1.9% at week 12, showing no statistically significant difference (p=0.4632). Fasting plasma glucose was 142 ± 45 and 180 ± 53 mg/dL, at week 0 and 12, respectively, also showing no statistically significant difference (p=0.1872). HbAlc in 15 patients not treated with insulin prior to the use of exenatide (non-insulin group) was 9.4 ± 1.3 % and 7.7 ± 1.2% at week 0 and 12, respectively, showing a significant decrease (p=0.0036). Fasting plasma glucose was 181 ± 43 and 155 ± 45 mg/dL at week 0 and 12, respectively, showing a significant decrease (p=0.0413).

Fasting CPR in the insulin group increased significantly from 1.48 ± 1.3 ng/mL at week 0 to 3.01 ± 1.32 ng/mL at week 12 (p=0.0008), while the non-insulin group showed no significant change from 2.59 ± 1.0 ng/mL to 2.82 ± 0.76 ng/mL (p=0.3074).

Body weight in the insulin group showed a significant decrease from 70.6 ± 8.4 kg at week 0 to 66 ± 9.2 kg at week 12 (p=0.0048), with a consequent decrease in BMI (27.9 ± 2.2 to 26.3 ± 1.8, p=0.0057). The non-insulin group also showed a significant decrease in body weight from 77.9 ± 15.7 kg to 74.4 ± 16.4 kg (p=0.0009), with a consequent decrease in BMI (29.5 ± 3.1 to 28.0 ± 3.3, p=0.001).

In 10 patients treated with DPP-4 inhibitor prior to the use of exenatide (DPP-4 inhibitor group), HbAlc showed a downward tendency (9.6 ± 0.9% to 8.4 ± 1.6%, p=0.0817) and fasting plasma glucose no significant change (185 ± 46 to 173 ± 54 mg/dL, p=0.5075).

In 14 patients not treated with DPP-4 inhibitor prior to the use of exenatide (non-DPP-4 inhibitor group), HbAlc was 9.0 ± 1.5% at week 0 and 7.6 ± 1.5% at week 12, showing a significant decrease (p=0.0324). Fasting plasma glucose in this group was not significantly changed (153 ± 44 to 158 ± 45 mg/dL, p=0.7977).

In the case of fasting CPR, no significant change was observed in the DPP-4 inhibitor group (2.79 ± 1.18 to 3.08 ± 1.47 ng/mL, p=0.3438), whereas a significant increase was observed in the non-DPP-4 inhibitor group (1.73 ± 1.1 to 2.75 ± 1.25 ng/mL, p=0.0031).

Body weight decreased significantly in the DPP-4 inhibitor group (78.0 ± 15.1 to 74.3 ± 16.1 kg, p=0.0096) with consequent decrease in BMI (29.2 ± 2.7 to 27.7 ± 3.1, p=0.0109). A significant decrease in boldy weight was also observed in the non-DPP-4 inhibitor group (73.1 ± 12.6 kg to 69.1 ± 13.1 kg, p=0.0004), with a consequent decrease in BMI (28.7 ± 3.0 to 27.1 ± 2.9, p=0.0005).

Discussion

In this study, we investigated the effects of exenatide on glycemic control, body weight, lipids, blood pressure, pulse rate, and gastrointestinal symptoms in obese Japanese patients with type 2 diabetes.

Assessment of the 24 patients treated for 12 weeks revealed a significant decrease in HbAlc by 1.5%. Body weight decreased and fasting serum CPR increased significantly. These results suggest that exenatide therapy not only decreases HbA1c by enhancing endogenous insulin secretion but also achieves weight reduction. Because nausea was observed in 14 patients (58%), the influence of gastrointestinal symptoms on plasma glucose levels and metabolic parameters was assessed by dividing the patients into two groups with or without nausea. Although body weight decreased significantly in both groups, HbAlc and GA decreased significantly only in the patients with nausea (Fig. 1a, 1b). In the patients without nausea, serum CPR increased significantly (Fig. 1d), but there was no improvement in plasma glucose (Fig. 1c).

Because there was no significant change in fasting

plasma glucose despite a significant decrease in HbAlc and improvement in GA, suppression of postprandial hyperglycemia seemed to be greater with exenatide treatment, although this effect appeared to be weaker in the patients without nausea. Improvement in glycemic control by exenatide was more difficult to achieve in the patients without nausea, most probably because of the influence of changes in postprandial hyperglycemia. The occurrence of nausea during exenatide administration is thought to be related to its action on the digestive tract through the vagus nerve; this action was presumably stronger in the patients with nausea than in those without. Suppression of gastrointestinal, particularly gastric, motility seems to control postprandial hyperglycemia. In addition, delayed gastric emptying may have led to decreased food intake and appetite suppression, resulting in better control of postprandial blood glucose levels.

In contrast, suppression of appetite was not observed in most patients without nausea. Accordingly, the reason for the weaker effect of exenatide on HbA1c and GA in the patients without nausea was thought to be weaker inhibition of gastrointestinal motility, leading to less improvement in postprandial hyperglycemia.

In both healthy subjects and patients with diabetes who receive exogenous GLP-1, delay of gastric emptying after liquid and solid meal intake has been reported [2, 3]. This effect is GLP-1-dependent and is also observed physiologically after food intake. The regulation of gastric emptying has a great influence on the postprandial blood glucose response, so whether gastric emptying is delayed by exenatide or not may have a marked difference of effect on postprandial blood glucose.

The timing of the onset of nausea, its duration, and the time of occurrence after the initiation of exenatide administration differed greatly among the patients, and these variations were assumed to affect postprandial blood glucose. When exenatide is initiated, attention should be paid to nausea, particularly for about 3 h after dosing and for about 10 days from the start of administration.

Assessment of all patients showed a significant increase in serum CPR, with an improvement in glycemic control (Fig. 1a, b, d). In contrast, improvement in glycemic control was not observed in the patients without nausea, although there was an increase in fasting serum CPR (Fig. 1d).

We assessed the effect of the treatment with insulin and DPP-4 inhibitor prior to the treatment with exanatide on plasma glucose and body weight. In the case of DPP-4 inhibitor, patients with and without the prior treatment showed similar decreases in fasting plasma glucose and body weight. In the case of insulin, similar improvement in body weight was observed in patients with and without the prior treatment. However, patients with the prior insulin treatment failed to show improvement in HbAlc with worsened values of fasting plasma glucose in spite of an increase in fasting CPR.

Three of 15 patients with nausea and 6 of 9 patients without nausea were found to belong to a group of patients who received prior treatment with insulin. The fact that a large number of nausea-free patients were found in the insulin group is likely related to an increase in fasting plasma glucose and no improvement in HbAlc in spite of an increase in fasting CPR. Improvement in HbAlc was observed in patients with nausea who showed no significant increase in fasting CPR, suggesting that improvement in glycemic control/plasma glucose levels by exenatide is due not only to promotion of endogenous insulin secretion but also to aforementioned delay in gastric emptying or suppression of glucagon secretion (not investigated in this study).

Exenatide administration also resulted in a significant decrease in systolic blood pressure and LDL cholesterol with improvement in blood pressure and lipid metabolism (Fig. 2c, 2d).

It has already been reported that exenatide improves lipid metabolism in patients with type 2 diabetes and patients with impaired glucose tolerance.

Control of sodium reabsorption in the proximal renal tubules [4], suppression of phosphorylation of extracellular signal-regulated kinase by angiotensin II in the kidneys [5], and improvement in vasodilation by increasing NO (nitric oxide) production in vascular endothelial cells [6] are considered to be the mechanisms underlying the antihypertensive action of exenatide.

A previous study into the effect of GLP-1 agonist therapy on lipid metabolism in patients with type 2 diabetes or impaired glucose tolerance also showed that exenatide improves lipid metabolism [7].

Improvement in systolic blood pressure and LDL cholesterol was more marked in the patients with nausea. The total food intake and sodium intake were not controlled during the 12-week administration period of this study, which is considered to be one potential reason for the significant improvement in lipids and blood

pressure only in the patients with nausea. It has been reported that LDL cholesterol is improved by long-term administration of exenatide [8]; therefore, a longer observation period is necessary in future studies.

It has been reported that obese people are often associated with characteristic eating behavior, such as overeating, lack of a sense of fullness, desire for fatty/lipid-rich foods and short mealtime [9]. In the present study, medical interviews revealed that patients treated with exenatide ate less snacks and fatty/lipid-rich foods and took a longer time for eating. These results suggest that changes in the amount of food, eating manner and favorite food play a role in improving body weight and blood pressure.

The body weight control could be a key factor for reduction of risks of cardiovascular events and diabetes complications. The present study indicates that exenatide decreases body weight and effectively improves both HbAlc and body weight particulary in patients with gastrointestinal symptoms.

Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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