management of dyslipidemia for prevention of CHD.

Currently approximately 4 million people are taking statins for hyperlipidemia in Japan. In this survey about 5% of the participants were taking lipid-lowering drugs, most of which are supposed to be statins. The mean total cholesterol level of the participants without lipid lowering drugs was 209 mg/dl, which is slightly higher than the mean total cholesterol levels of all the participants. In this sense, the participants in this survey represent the general population in Japan. Use of lipid-lowering drugs such as statins would be more important for the treatment of high risk patients to prevent CHD.

In 2000, another survey was conducted by the Ministry of Health, Labor, and Welfare. In this study, more subjects were selected from rural, agricultural, and mountainous areas, and the results showed no rise in serum cholesterol in the last 10 years (from 1990 to 2000). In this study carried out by the members of the Japan Atherosclerosis Society, more subjects from urban areas were included. In both studies, the cholesterol levels were significantly lower in the agricultural and mountainous districts than in the districts including large cities like Tokyo and Osaka in 1980. In 1990, the difference in serum cholesterol levels was no longer significant between urban, rural, and mountain village areas. Therefore, it is not clear why these studies show a different trend in the cholesterol level. However, Kuzuya et al also found an increase in total cholesterol levels from 1989 to 1998 in Aichi Prefecture in the central region of Japan (21).

In this survey we also determined fasting glucose, insulin, and HbA1c levels of approximately 10,000 participants. We think that this is the largest survey of glucose metabolism in Japan. Our data indicate that the glucose and HbA1c levels gradually increased according to age in both sexes. However, the plasma insulin levels are almost constant in all age groups. We also showed that the uric acid level was significantly higher in men than in women. This is consistent with the data that the incidence of hyperuricemia and gout is higher in males than in females. Alcohol consumption would contribute to the higher level of uric acid in men. According to the database from the Ministry of Health, Labor, and Welfare (http://www.mhlw.go.jp/toukei/), the incidence of hyperuricemia in men and women is increasing in Japan. Because hyperuricemia is related to obesity, hypertension, and insulin resistance, and eventually to the incidence of CHD, controlling the uric acid level would be important for the prevention of CHD in Japan.

Thus this report tells us the importance of the prevention and treatment of hyperlipidemia for the prevention of CHD in Japan. We need to establish guidelines for lifestyle change to prevent the further increase of dyslipidemia in the future.

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Appendix

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Critical Roles of CXC Chemokine Ligand 16/Scavenger Receptor that Binds Phosphatidylserine and Oxidized Lipoprotein in the Pathogenesis of Both Acute and Adoptive Transfer Experimental Autoimmune Encephalomyelitis[1]

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The scavenger receptor that binds phosphatidylserine and oxidized lipoprotein (SR-PSOX)/CXCL16 is a chemokine expressed on macrophages and dendritic cells, while its receptor expresses on T and NK T cells. We investigated the role of SR-PSOX/CXCL16 on acute and adoptive experimental autoimmune encephalomyelitis (EAE), which is Th1-polarized T cell-mediated autoimmune disease of the CNS. Administration of mAb against SR-PSOX/CXCL16 around the primary immunization decreased disease incidence of acute EAE with associated reduced infiltration of mononuclear cells into the CNS. Its administration was also shown to inhibit elevation of serum IFN-γ level at primary immune response, as well as subsequent generation of Ag-specific T cells. In adoptive transfer EAE, treatment of recipient mice with anti-SR-PSOX/CXCL16 mAb also induced not only decreased clinical disease incidence, but also diminished traffic of mononuclear cells into the CNS. In addition, histopathological analyses showed that clinical development of EAE correlates well with expression of SR-PSOX/CXCL16 in the CNS. All the results show that SR-PSOX/CXCL16 plays important roles in EAE by supporting generation of Ag-specific T cells, as well as recruitment of inflammatory mononuclear cells into the CNS. *The Journal of Immunology,* **2004, 173: 1620–1627.**

Multiple sclerosis (MS)[3] and its animal model, experimental autoimmune encephalomyelitis (EAE), are type I (Th1)-polarized T cell-mediated autoimmune diseases of the CNS (1, 2). In both diseases, Th1 autoreactive T cells for self-Ags in the CNS are initially generated (3, 4), and then circulating leukocytes including the autoreactive T cells penetrate the blood brain barrier. Finally, the penetrated leukocytes induce damage of myelin, resulting in impaired nerve conduction and paralysis (2). However, the mechanisms of generation of autoreactive Th1 T cells, recruitment of leukocytes into the CNS, and accumulation of the leukocytes in the CNS before and during clinical disease are not well understood.

Chemokines are a family of cytokines exhibiting selective chemoattractant properties for target leukocytes. Based on the motif of the first two cysteines, chemokines have been classified into four highly conserved but distinct subfamilies: CC, CXC, C, and CX3C chemokines (5). Chemokines play an important role in recruitment of leukocytes at the site of initial immunoreactions induced by such as infection as well as infiltration of leukocytes into the site of inflammation during the T cell-mediated inflammatory conditions (3, 6). Various chemokine members have been implicated as candidates involved in the immunopathology of EAE. CCL2/MCP-1, CCL3/MIP-1α, and CXCL1/IFN-γ-inducible protein-10 (IP-10) were reported to be expressed in the CNS in acute rat and murine EAE models (7, 8). T cell clones, which could induce adoptive transfer EAE, were reported to express CCR5, a receptor for CCL3/MIP-1α.

MIP-1α was demonstrated to play a functionally significant role in pathogenesis of mouse EAE by analyses that administration of neutralizing polyclonal Ab for MIP-1α suppressed severity of clinical EAE through partial inhibition of recruitment of inflammatory mononuclear cells including Th1 T cells into the CNS (7). In the case of IP-10, two contradictory results were reported (9, 10). Administration of neutralizing Ab against IP-10 partially decreased or increased clinical and histological disease incidence and severity, as well as infiltration of mononuclear cells into the CNS. As for MCP-1, gene-disrupted mice of MCP-1 and its receptor, CCR2, were shown to be completely resistant to development of EAE by inability of monocyte to be recruited into the CNS (9, 11, 12). Recruitment of monocytes into the CNS by MCP-1 must play an essential rule in EAE, while it has not been clear whether chemokines other than MIP-1α and IP-10 are involved in the infiltration of autoreactive T cells into the CNS.

Recently, polyclonal Ab against CCL20/MIP-3α was reported to partially suppress clinical EAE by inhibiting sensitization of naive lymphocytes to myelin Ags through inhibition of naive dendritic cells (DCs) trafficking or by inhibiting exit of sensitized lymphocytes from the draining lymph nodes (13). However, it has not

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[3] Abbreviations used in this paper: MS, multiple sclerosis; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; IP-10, IFN-γ-inducible protein-10; MOG, myelin oligodendrocyte glycoprotein; SR-PSOX, scavenger receptor that binds phosphatidylserine and oxidized lipoprotein.

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been clear what chemokines are involved in the generation of Th1-polarized autoreactive T cells in primary immune response in EAE.

Recently, we and others identified a novel transmembrane protein that was designated as SR-PSOX (scavenger receptor that binds phosphatidylserine and oxidized lipoprotein) and CXCL 16, respectively (14–16). Interestingly, SR-PSOX/CXCL16 was shown to possess two different biological activities, scavenger receptor and chemokine activities. SR-PSOX/CXCL16 is the ligand for Bonzo/CXCR6 expressed on naive and active CD8 T cells, Th1-polarized activated CD4, and naive and activated NK T cells (14, 15). SR-PSOX/CXCL16 was shown to have chemoattractant activity for activated T cells, but not naive CD8 T cells (15). Cell surface-anchored SR-PSOX/CXCL16 with a transmembrane domain shows not only scavenger receptor activity but also cell adhesion activity against CXCR6-expressing cells, while membrane metalloprotease-cleaved soluble SR-PSOX/CXCL16 shows chemokine activity for CXCR6-expressing cells (17, 18). Bonzo/CXCR6, a receptor of SR-PSOX/CXCL16, was reported to be expressed on a subset of Th1 T cells but not on Th2 T cells, and its expression has been regarded as a differential marker of Th1-polarized T cells (19). Furthermore, expression of Bonzo/CXCR6 was confirmed in myelin basic protein-reactive T cell lines with IFN-γ-producing activity (20).

In this study, we investigated in vivo effects of neutralizing anti-SR-PSOX/CXCL16 mAb in both acute and adoptive transfer EAE. SR-PSOX/CXCL16 was clearly shown to play an important role in different two phases of EAE: IFN-γ-production at primary immune response followed by generations of myelin basic protein-specific T cells and recruitment of mononuclear cells into the CNS.

Materials and Methods

Mice and Ag

C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan) and housed under the specific pathogen-free condition. Myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}; MEVGWYRSPFSRVVHLYRNGK), which was used for Ags inducing acute EAE, was synthesized by Toray Research Center (Kamakura, Japan), and purity was determined to be >95% by reversed-phase HPLC.

Anti-mouse SR-PSOX/CXCL16 mAbs

We generated anti-SR-PSOX/CXCL16 mAb IgG1 12-81 as described previously (18). This Ab did not cross any other chemokines, which could be expected. Anti-human SR-PSOX mAb IgG1 22-19-12 was generated and characterized as described previously (17). Both mAbs were provided by Sankyo (Tokyo, Japan).

Chemotaxis and calcium mobilization assays

Chemotaxis assay using transwell plates with 5-μm pore size membrane (Corning Japan, Tokyo, Japan) and calcium mobilization assays were performed as described previously (18, 21).

Effect of anti-SR-PSOX/CXCL16 mAb on soluble SR-PSOX/CXCL16-binding to CXCR6-expressing cells

CXCR6-expressed L1.2 cells (17) were incubated for 1 h on ice with mouse SR-PSOX/CXCL16-Fc (1 μg/ml), which was preincubated with anti-SR-PSOX mAbs 12-81 (5 μg/ml) or control rat IgG for 30 min on ice. For determining the quantity of cell-bound SR-PSOX/CXCL16-Fc, cells were stained with PE-labeled goat anti-human-Fc and analyzed by flow cytometry using Epics XL (Beckman Coulter, Fullerton, CA).

Expression analysis of CXCR6 on spleen T cells

Spleen cells were prepared from mice on day 4 after immunization of MOG_{35-55}, and cultured for 4 days in the presence or absence 25 μg/ml MOG_{35-55}. Cells were incubated for 1 h on ice with mouse SR-PSOX/CXCL16-Fc (1 μg/ml), followed by staining with PE-labeled goat anti-human-Fc, together with FITC-labeled anti-mouse CD4 mAb or FITC-labeled anti-mouse CD8 mAb (BD Biosciences, San Diego, CA) as described previously (18), and then analyzed by flow cytometry.

Active induction of EAE with MOG peptides and clinical evaluation

Six to 9-wk-old female mice were immunized by s.c. injection into thighs of bilateral hind feet with 150 μg/mouse of MOG peptides in 0.15 ml of sterilized PBS emulsified with an equal volume of CFA containing 4 mg/ml *Mycobacterium tuberculosis* (BD Diagnostic Systems, Sparks, MD) (22, 23). Two hours before and 2, 4, and 7 days after the immunization, mice were injected with 500 μg anti-SR-PSOX/CXCL16 mAb or control rat IgG. The mice were i.v. injected with pertussis toxin (List Biological Laboratories, Campbell, CA) at day 0 and 2 after immunization. Animals were scored daily for 5 wk for clinical signs of EAE using the following criteria: 0, no clinical signs; 1, limp tail (tail paralysis); 2, complete loss of tail tonicity or abnormal gait; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, forelimb paralysis or moribund; and 6, death (24).

Passive transfer of EAE

Spleen-cell suspension was prepared from mice 4 days after the immunization with MOG_{35-55} as described in acute EAE and cultured in RPMI 1640 containing 10% FCS and 25 μg/ml MOG_{35-55} for 4 days at 37°C. Cells were harvested, washed three times with PBS and transferred i.v. into normal C57BL/6 recipient mice ($1–2 \times 10^7$ viable cells/mouse). Animals were scored daily for 5 wk for signs of EAE using the above-described criterion (8, 13).

T cell proliferation assay

Spleen-cell suspension (5×10^6/ml) was prepared from mice on day 6 after immunization of MOG_{35-55}, and cultured in triplicate in 96-well flat-bottom plates (IWAKI, Tokyo, Japan) in the presence or absence of MOG_{35-55}. Cells were pulsed with 0.5 μCi/well [^{3}H]thymidine (Valeant Pharmaceuticals, Costa Mesa, CA) at 72 h after cultivation, and incubated further for 12 h. Cells were then harvested and incorporated radioactivity was measured using a MicroBeta PLUS liquid scintillation counter with software v3.3 (PerkinElmer Wallac, Gaithersburg, MD).

Quantification of IFN-γ by ELISA

IFN-γ in serum, which was prepared from MOG_{35-55}-immunized mice on 36, 48, 60, and 84 h after immunization, was quantified using a mouse IFN-γ ELISA kit (GE Medical Systems, Milwaukee, WI), and the data were measured using Wallac 1420 ARVO fluoroscan (PerkinElmer Wallac).

Spleen-cell suspension (5×10^6/ml) was prepared from mice on day 6 after immunization of MOG_{35-55}, and cultured in triplicate in 96-well flat-bottom plates (IWAKI) in the presence or absence of MOG_{35-55} for 48 h. IFN-γ in the culture supernatants was quantified using a mouse IFN-γ ELISA kit (Amersham Biosciences), and the data were measured using Wallac 1420 ARVO fluoroscan (PerkinElmer Wallac).

RT-PCR for SR-PSOX/CXCL16 and CXCR6 mRNA

Total RNA was extracted from PBS-perfused and snap-frozen spinal cords using a total RNA Purification System (Invitrogen Life Technologies, Carlsbad, CA). cDNA was synthesized and RNA was amplified using Ready-To-Go RT-PCR Beads (GE Medical Systems). CXCR6 was detected by using the forward primer, 5′-CACTCTGGAACAAAGCTACTGGGCT-3′, and reverse primer, 5′-AGGTGAGAGTGAGCATGGACA-3′, and CXCL16 was detected by using primers as previously described (14).

Histological assessment for EAE

Spinal cords from EAE-induced mice were dissected on day 15 after immunization and fixed in 4% neutral buffered formalin in PBS. Paraffin-embedded sections of 4-μm thickness were cut from the spinal cords and stained with H&E or with the myelin-specific Bodian and Luxol fast blue (25). For in situ hybridization, paraffin-embedded sections of spinal cords from EAE-induced mice were sliced and fixed on glass slides precoated with 3-aminopropyltriethoxysilane. Antisense and sense ^{35}S-labeled cDNA probes specific for mouse SR-PSOX/CXCL16 were prepared by in vitro transcription with T3/T7 RNA polymerase (Stratagene, La Jolla, CA). SR-PSOX/CXCL16 mRNA-positive cells were examined under fluorescence microscope. Data were collected in several independent visual fields (24).

Results

Characterization of anti-SR-PSOX/CXCL16 mAb 12-81

To characterize rat anti-mouse SR-PSOX/CXCL16 mAb 12-81, we examined inhibitory activity of the mAb against the chemotactic activity of soluble SR-PSOX/CXCL16 for CXCR6-expressing cells using the standard transwell assay. The number of cells that migrated into bottom wells was shown to decrease in accordance with the concentration of anti-SR-PSOX/CXCL16 mAb (Fig. 1*A*). Then, we examined whether the mAb inhibits direct binding of SR-PSOX/CXCL16 to its receptor, CXCR6. Direct binding of soluble SR-PSOX/CXCL16-Fc to CXCR6-expressing L1.2 cells was quantified by flow cytometry. Anti-SR-PSOX/CXCL16 mAb 12-81 was clearly shown to specifically inhibit direct binding of SR-PSOX/CXCL16-Fc to CXCR6-expressing cells (Fig. 1*B*). These results indicate that anti-SR-PSOX/CXCL16 mAb 12-81 can inhibit SR-PSOX/CXCL16-induced migration as well as direct binding of SR-PSOX/CXCL16 to CXCR6-expressing cells.

Anti-SR-PSOX/CXCL16 mAb-treated mice are resistant to induction of acute EAE

To determine the roles of SR-PSOX/CXCL16 on development of clinical EAE, we examined the in vivo activity of neutralizing anti-mouse SR-PSOX/CXCL16 mAb 12-81 against induction of clinical acute EAE. Mice, immunized with the encephalitogenic peptide MOG_{35-55} on day 0, were administrated with anti-SR-PSOX/CXCL16 mAb on day 0, 2, 4, and 7, and scored daily for signs of disease (Fig. 2*A*). As expected, mice treated with control rat IgG instead of anti-SR-PSOX/CXCL16 mAb developed clinical EAE with a mode incidence of 100%. In contrast, the clinical disease severity was dramatically decreased in anti-SR-PSOX/CXCL16 mAb-treated mice. Although the control mice developed severe EAE with a mean clinical score of 3.8 on day 18 after immunization, anti-SR-PSOX/CXCL16 mAb-treated mice developed disease with a significantly decreased mean clinical score of 1.3 on day 19 with associated delayed onset of the clinical disease. Interestingly, administration of anti-SR-PSOX/CXCL16 mAb on only day 0 and 2 after immunization of MOG_{35-55} was shown to be enough to induce delayed onset of clinical EAE while reduction of the disease severity was lower than administration on day 0, 2, 4, and 7 after immunization (data not shown). Administration of anti-SR-PSOX/CXCL16 mAb on day 4 and 7 after immunization did not affect clinical EAE. All the results indicate that in vivo neutralization of SR-PSOX/CXCL16 before the onset of clinical EAE significantly reduced the severity as well as early onset of acute EAE.

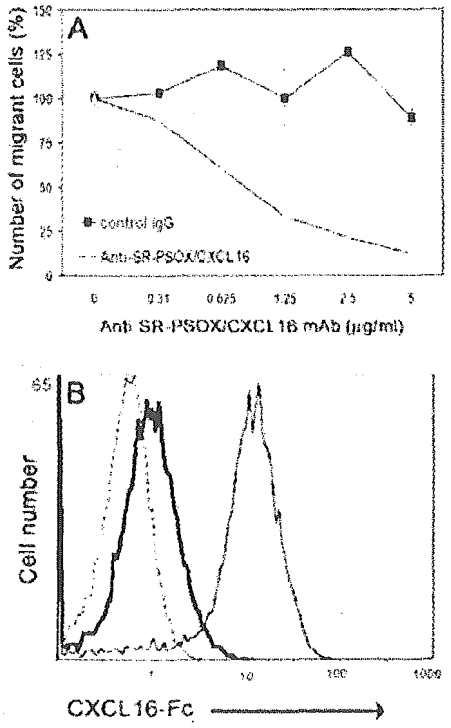

FIGURE 1. Characterization of anti-SR-PSOX/CXCL16 mAb 12-81. *A*, Effect on SR-PSOX/CXCL16-induced chemotaxis. Migration of CXCR6-expressing L1.2 cells to recombinant soluble SR-PSOX/CXCL16 (100 ng/ml) was quantified by chemotaxis assay using transwell plate as described previously (21). Neutralizing activity of anti-SR-PSOX/CXCL16 was analyzed by using CXCR6-expressing cells pretreated with indicated amounts of anti-SR-PSOX/CXCL16 mAb 12-81 or control rat IgG. Values in the absence of Ab were set as 100%. The data shown represent the mean ± SD ($n = 3$). *B*, Effect of SR-PSOX/CXCL16-binding to CXCR6-expressing cells. Binding of control Fc (dotted line) or soluble SR-PSOX/CXCL16-Fc (solid line) against CXCR6-expressing L1.2 cells was analyzed by flow cytometry. SR-PSOX/CXCL16-Fc was preincubated with control rat IgG (thin line) or anti-SR-PSOX/CXCL16 mAb 12-81 (bold line), as described in *Materials and Methods*.

Paucity of demyelinating lesions with inflammation in EAE mice treated with anti-SR-PSOX/CXCL16 mAb

Then, we histologically examined spinal cords of acute EAE-induced mice treated with or without anti-SR-PSOX/CXCL16 mAb (Fig. 2, *B–E*). Anti-SR-PSOX/CXCL16 mAb-treated mice showed no signs of histological EAE with demyelination and mononuclear cell infiltration, while there were numerous inflammatory and demyelinating lesions in the perivascular areas and parenchymal in spinal cord of control rat IgG-received mice that showed severe clinical EAE. These inflammatory lesions contain infiltrated mononuclear cells, while spinal cords of anti-SR-PSOX/CXCL16 mAb-treated mice lacked mononuclear cell infiltration and showed significantly decreased number of demyelinating lesions per section compared with the control mice. These results of histological analyses in anti-SR-PSOX/CXCL16 mAb-received mice correlate well with the absence of clinical EAE. Thus, inhibition of SR-PSOX/CXCL16 activities by anti-SR-PSOX/CXCL16 mAb 12-81 is efficient to suppress both clinical and histological EAE.

Decrease of MOG_{35-55}-specific T cell response in anti-SR-PSOX/CXCL16 mAb-treated mice

Because anti-SR-PSOX/CXCL16 mAb-treated mice were resistant to development of both clinical and histological EAE, we examined which phase was inhibited by administration of anti-SR-PSOX/CXCL16 mAb 12-81, generation of MOG_{35-55}-specific Th1-polarized T cells, or infiltration of MOG_{35-55}-specific T cells into the CNS. We analyzed generation of Ag-specific T cells in spleens of MOG_{35-55}-immunized mice. Spleen cells were prepared from mice on day 6 after immunization with MOG_{35-55}, and their MOG_{35-55}-specific in vitro proliferation and in vitro IFN-γ production were quantified by [^{3}H]thymidine incorporation assay (Fig. 3*A*) and by ELISA (Fig. 3*B*), respectively. Spleen T cells from control rat IgG-received mice showed robust and dose-dependent proliferative response in accordance with the amounts of added MOG_{35-55}. In contrast, spleen T cells from anti-SR-PSOX/CXCL16 mAb-treated mice did not show MOG_{35-55}-dependent increase of cell proliferative response in vitro (Fig. 3*A*). Moreover, MOG_{35-55}-dependent increase of in vitro IFN-γ production was not induced in spleen cells from anti-SR-PSOX/CXCL16 mAb-received mice, although MOG_{35-55}-induced dramatic increase of

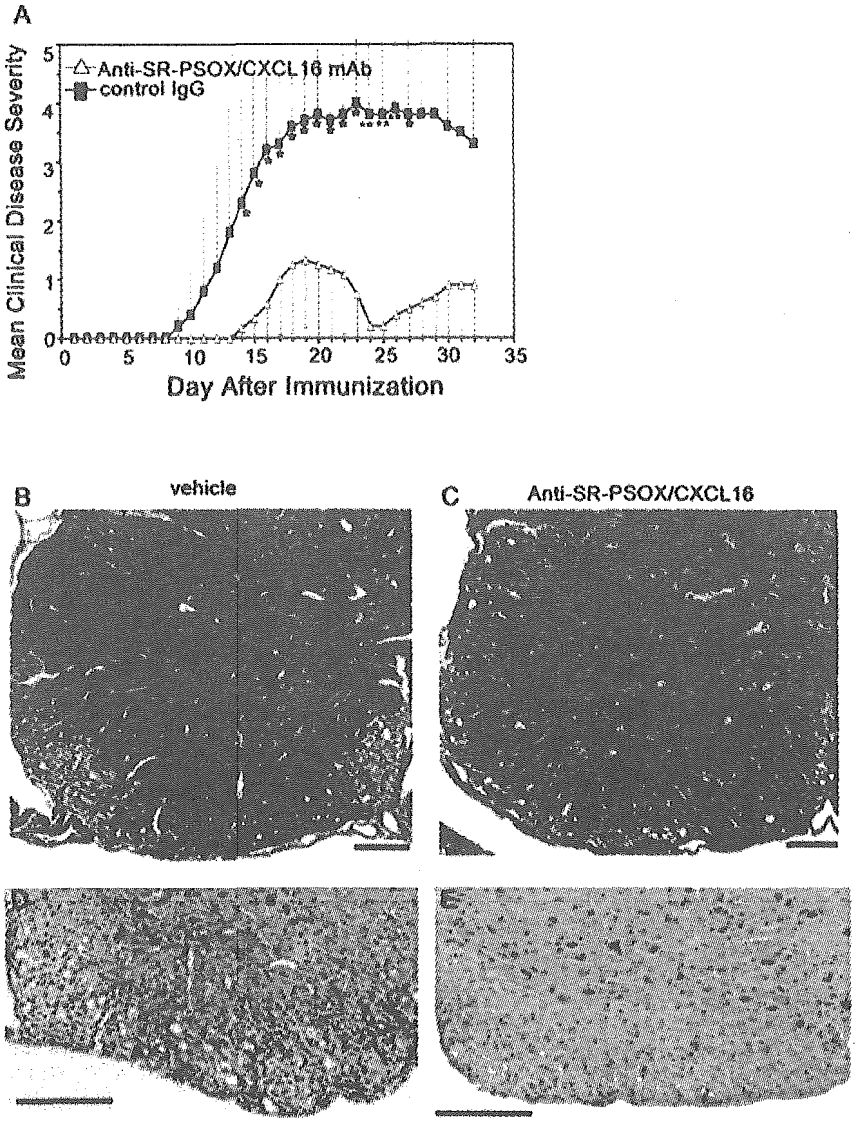

FIGURE 2. Effects of anti-SR-PSOX/CXCL16 mAb 12-81 on development of clinical disease in MOG_{35-55}-induced acute EAE. *A*, Development of MOG_{35-55}-induced clinical EAE. Mice were immunized with MOG_{35-55} and then received pertussis toxin as described in *Materials and Methods*. Results of mice receiving control rat IgG (n = 7) or anti-SR-PSOX/CXCL16 mAb (n = 7) are expressed as mean disease score ± SD. The data are representative of three independent experiments with essentially similar results; *, $p < 0.02$; **, $p < 0.01$ (Mann-Whitney U test). *B–E*, Histological examination. Spinal cords from EAE-induced mice treated with control rat IgG (*B* and *D*) or anti-SR-PSOX/CXCL16 mAb (*C* and *E*) were analyzed. Serial tissue sections were stained by Bodian and Luxol fast blue (*B* and *C*) and H&E (*D* and *E*) in each mouse. Demyelinating lesions (*B*) and lesions with infiltrated mononuclear cells (*D*) are indicated in control IgG-treated mice, while little demyelinating lesion (*C*) and lesions with only some extent of perivascular mononuclear cell infiltration (*E*) are indicated in anti-SR-PSOX/CXCL16 mAb-treated mouse. Scale bars, *B* and *C*, 150 μm; *D* and *E*, 200 μm.

IFN-γ production was observed in spleen cells from control rat IgG-received mice (Fig. 3*B*). These results suggest that generation of MOG_{35-55}-specific T cells was inhibited by anti-SR-PSOX/CXCL16 mAb in primary in vivo immune response, because mice received MOG_{35-55} only once on day 0.

Because EAE is a Th1-dominant autoimmune disease model, we also examined the amount of proinflammatory Th1 cytokine IFN-γ secreted in serum of mice immunized with MOG_{35-55}. IFN-γ is known to be involved in the pathogenesis of EAE at different points in the course of disease, including primary immune response (26, 27). At 48 and 60 h after primary immunization with MOG_{35-55}, amount of secreted IFN-γ was elevated in serum of mice receiving control rat IgG. In contrast, production of IFN-γ was vigorously suppressed in mice receiving anti-SR-PSOX/CXCL16 mAb (Fig. 3*C*). These observations suggest that anti-SR-PSOX/CXCL16 mAb inhibits generation of MOG_{35-55}-specific Th1 T cell by suppression of IFN-γ production in primary immune response.

Anti-SR-PSOX/CXCL16 mAb-treated mice are resistant to induction of adoptive transfer EAE

Next, we examined whether anti-SR-PSOX/CXCL16 mAb 12-81 could inhibit migration of MOG_{35-55}-specific activated T cells into the spinal cord. Then, effect of administrated anti-SR-PSOX/CXCL16 mAb was analyzed in adoptive transfer EAE, which is developed in recipient mice receiving MOG_{35-55}-specific activated T cells. Anti-SR-PSOX/CXCL16 mAb-treated mice receiving MOG_{35-55} peptide-specific T cells were indicated to manifest significantly delayed onset of clinical EAE at day 21 on average, while control rat IgG-treated mice showed onset of clinical EAE at day 13 on average (Fig. 4). In addition, anti-SR-PSOX/CXCL16 mAb-treated mice showed milder neurological impairment in histological EAE (data not shown). These results suggest that SR-PSOX/CXCL16 is relevant to onset of adoptive transfer EAE by inducing migration of MOG_{35-55}-specific T cells into CNS, because CXCR6, a receptor of SR-PSOX/CXCL16, was reported to be expressed on activated $CD4^+$, and naive and activated $CD8^+$ T cells (14, 15).

Then, we analyzed whether CXCR6 was actually expressed on activated T cells used in adoptive transfer EAE (Fig. 5). Although a small part of $CD4^+$ spleen T cells from unimmunized mice expressed increased amounts of CXCR6 after in vitro cultivation with MOG_{35-55}, the number of CXCR6-positive $CD4^+$ T cells was significantly larger in splenocytes of MOG_{35-55}-immunized mice than in unimmunized mice after in vitro cultivation with

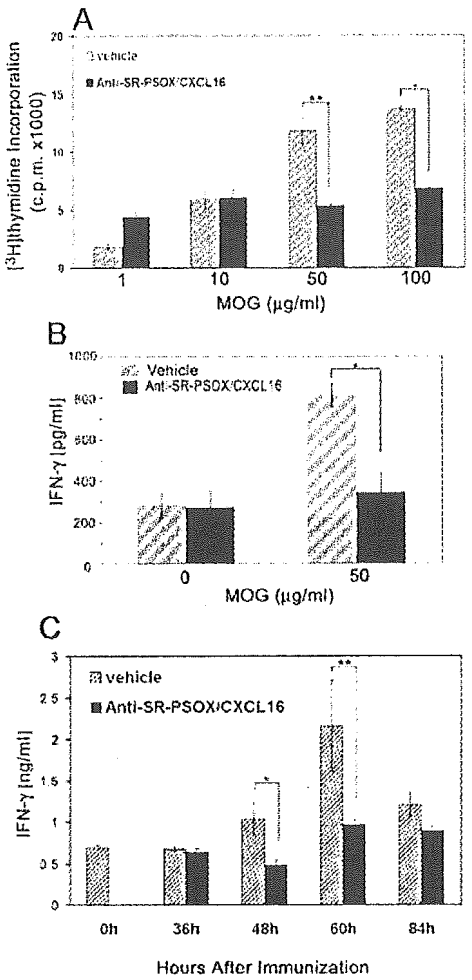

FIGURE 3. Effects of anti-SR-PSOX/CXCL16 mAb 12-81 on MOG_{35-55}-specific immune responses. *A* and *B*, Six days after administration of MOG_{35-55}, splenocytes were isolated from mice treated with anti-SR-PSOX/CXCL16 mAb (anti-SR-PSOX/CXCL16) or control rat IgG (vehicle). *A*, Incorporation of [^{3}H]thymidine by the splenocytes was measured after in vitro stimulation with MOG_{35-55}-peptide as described in *Materials and Methods*; *, $p < 0.002$; **, $p < 0.02$. *B*, Production of IFN-γ by the splenocytes was measured after in vitro stimulation with MOG_{35-55}-peptide as described in *Materials and Methods*; *, $p < 0.03$. *C*, Production of IFN-γ was quantified by ELISA in serum of MOG_{35-55}-injected mice treated with anti-SR-PSOX/CXCL16 mAb (anti-SR-PSOX/CXCL16) or rat IgG (vehicle). Serum was harvested at indicated hours after the immunization with MOG_{35-55}; *, $p < 0.002$; **, $p < 0.02$.

MOG_{35-55}. However, in $CD8^+$ spleen T cells, in vitro cultivation with MOG_{35-55} similarly increased expression of CXCR6 between MOG_{35-55}-immunized and -unimmunized mice. Thus, in vitro cultivation with MOG_{35-55} specifically augments expression of CXCR6 on $CD4^+$ T cells from MOG_{35-55}-immunzed mice, which may be relevant to onset and development of transfer EAE.

Expression of SR-PSOX/CXCL16 in spinal cord of EAE mice

Then, we ascertained the expression of SR-PSOX/CXCL16 in the CNS of EAE mice that might induce chemotaxis of MOG_{35-55}-specific activated T cells into the CNS. Expression of SR-PSOX/CXCL16 was analyzed in the spinal cords of EAE mice 14 days after immunization by in situ hybridization. SR-PSOX/CXCL16-expressing cells were observed around in the white matter of the spinal cord, while significant expression of SR-PSOX/CXCL16

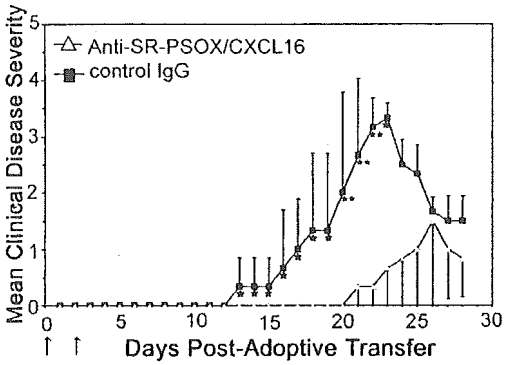

FIGURE 4. Effects of anti-SR-PSOX/CXCL16 mAb 12-81 on onset and development of adoptive transferred clinical EAE. Mice were immunized with MOG_{35-55} as described in Fig. 2, and splenocytes of the mice 4 days after the immunization were cultured with MOG_{35-55} for 4 days, and then, obtained MOG_{35-55}-specific T cell blasts were transferred into recipient B6 mice as described in *Materials and Methods*. Two hours before and 2 days after the transfer of encephalitogenic T cells, recipient mice were injected i.p. with 0.2 ml/mouse of PBS containing 500 μg of anti-SR-PSOX/CXCL16 mAb or control rat IgG (arrows). The data of mice receiving anti-SR-PSOX/CXCL16 mAb ($n = 6$) or control rat IgG ($n = 6$) are expressed as the mean clinical disease score ± SD; *, $p < 0.05$; **, $p < 0.002$. The data are representative of three independent experiments with similar results.

was not detected in control mice (Fig. 6, *B* and *C*). Next, we confirmed the expression of SR-PSOX/CXCL16 by RT-PCR in spinal cords of EAE mice. SR-PSOX/CXCL16 mRNA was shown to express at low level in spinal cords of mice 2, 4, and 6 days after immunization with MOG_{35-55}, while at detectable high level after onset of acute clinical EAE 14 days after immunization (Fig. 6*D*). The results suggested that a small increase of SR-PSOX/CXCL16 mRNA in the spinal cords preceded the development of clinical disease symptoms, which might have an affect on the disease onset, and its high expression was induced after disease onset.

From the observation that SR-PSOX/CXCL16 was highly produced in the CNS at the time of clinical EAE onset, we predicted that T cells in the CNS after induction of EAE would express CXCR6, the receptor for SR-PSOX/CXCL16. To ascertain this possibility, we analyzed CXCR6 mRNA level by RT-PCR in the spinal cords of EAE-induced mice. CXCR6 was shown to express in the CNS of acute clinical EAE 14 days after immunization, while expression of CXCR6 was not detected in mice 2, 4, and 6 days after immunization of MOG_{35-55} before onset of clinical EAE (Fig. 6*D*). Taken together, both SR-PSOX/CXCL16 and its receptor CXCR6 were expressed in the CNS during the development of acute EAE.

Discussion

EAE is a Th1-polarized T cell-mediated autoimmune disease of the CNS in appropriate strains of laboratory animals with a relapsing-remitting course that serves as a model for human MS. Th1-poralized T cells that are reactive to autoantigen in the CNS play an essential role in development of EAE (1, 2, 4). In EAE induced in this manuscript, such autoreactive Th1-poralized T cells were generated in peripheral lymphoid organ by s.c. immunization with MOG_{35-55} peptides, and EAE was caused by infiltration of mononuclear cells including the autoreactive T cells into the CNS. The present study demonstrates that administration of neutralizing mAb against transmembrane chemokine SR-PSOX/CXCL16 suppresses the development of both acute and adoptive transfer EAE by inhibiting the generation of the MOG_{35-55}-reactive T cells in

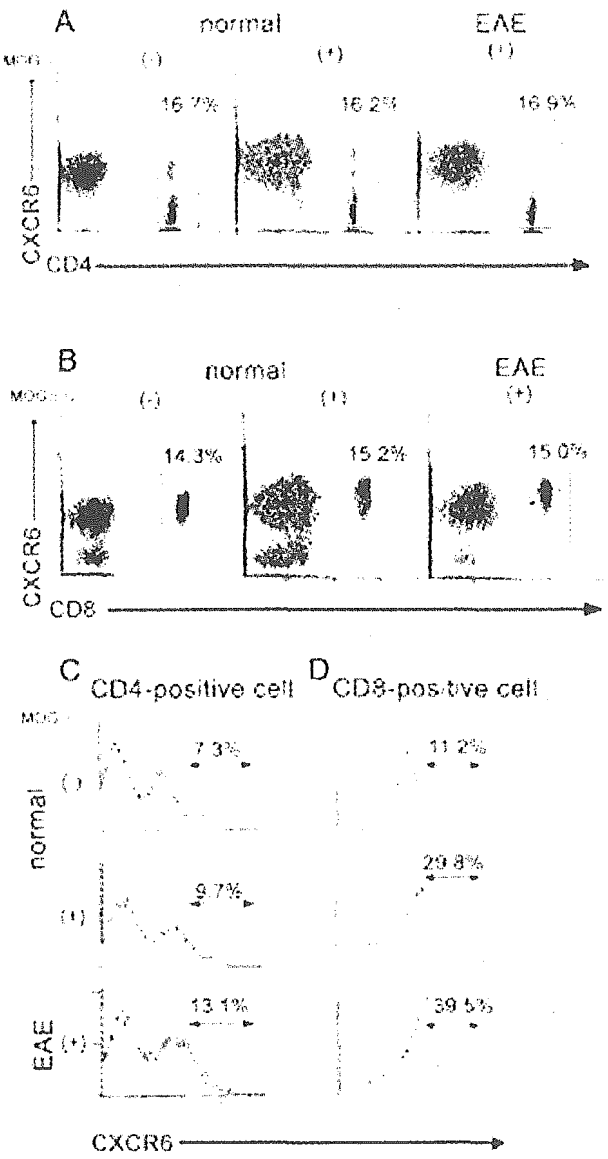

FIGURE 5. Expression of CXCR6 on MOG_{35-55}-specific activated CD4-positive and CD8-positive T cells. Mice were immunized with MOG_{35-55} as described in Fig. 2, and spleen cells of mice 4 days after immunization (EAE) or without immunization (normal) were analyzed before (−) and after (+) in vitro cultivation with MOG_{35-55} for 4 days. Cells were stained with SR-PSOX/CXCL16-Fc, followed by PE-labeled anti-human-Fc mAb together with anti-FITC-labeled CD4 mAb (*A* and *C*) or anti-FITC-labeled CD8 mAb (*B* and *D*), and then analyzed by two-dimensional flow cytometry (*A* and *B*). CD4-positive and CD8-positive cells in *A* and *B*, respectively, were gated, and the expression of CXCR6 was analyzed (*C* and *D*). The data are representative of three independent experiments with similar results.

periphery and the recruitment/accumulation of mononuclear cells, including MOG_{35-55}-reactive T cells, into the CNS, respectively. Thus, SR-PSOX/CXCL16 was suggested to play an important role in the development of EAE at different two phases: 1) generation of autoreactive Th1-polarized T cells and 2) its recruitment into and/or accumulation in the CNS.

In acute EAE, it was shown that SR-PSOX/CXCL16 was involved in generation of MOG_{35-55}-reactive T cells as well as production of IFN-γ in primary immune response against MOG_{35-55} peptides used as immunogen (Fig. 3). Because SR-PSOX/CXCL16 has been expressed on APCs such as DCs and macrophages (14, 15, 17), which play an important role in primary immune response, SR-PSOX/CXCL16 may be involved in generation of Th1-polarized T cells through supporting IFN-γ production in primary immune response. However, it has not been clarified which activity of SR-PSOX/CXCL16, scavenger receptor or chemokine activity, plays a role in generation of the MOG-reactive T cells, although it is possible that both activities coordinately function on the generation of the MOG-reactive T cells. As a scavenger receptor, SR-PSOX/CXCL16 on APCs may be able to support uptake and presentation of various Ags including MOG peptide. As a transmembrane chemokine, SR-PSOX/CXCL16 exerts chemotaxis-inducing activity against CXCR6-expressing cells after cleaving by membrane metalloprotease (18). CXCR6 was shown to express on naive CD8 T cells, Th1-polarized activated CD4 T cells, and naive and activated NK T cells (15). SR-PSOX/CXCL16 on DCs may play a role to induce chemotaxis of T and/or NK T cells. We are now analyzing a role of NK T cells in generation of the MOG-reactive T cells, because NK T cells with high expression of CXCR6 have been reported to support primary Th1-poralized immune response by producing high amounts of IFN-γ (28, 29). Involvement of NK T cells in the generation of Th1-inclined autoreactive T cells in EAE must be intimately examined in the near future.

In adoptive transfer EAE, SR-PSOX/CXCL16 was indicated to induce mononuclear cell traffic into the CNS. In addition, our data indicate that clinical disease severity of both acute and adoptive transfer EAE correlates well with expression levels of both SR-PSOX/CXCL16 and its receptor CXCR6 in spinal cords (Fig. 6*D*). Because 1) CXCR6 was expressed on both $CD4^+$ and $CD8^+$ T cells used in transfer EAE (Fig. 5); 2) CXCR6 was reported to express on a subset of Th1-polarized T cells but not on Th2 T cells or monocytes/macrophages (19); 3) SR-PSOX/CXCL16 was reported to have chemoattractant activity for activated T cells but not for monocytes/macrophages (15, 17); and 4) EAE has been regarded as Th1 T cell-mediated autoimmune disease (1, 2), SR-PSOX/CXCL16 may induce chemotaxis of MOG_{35-55}-specific Th1-polarized activated CD4 T cells and/or CD8 T cells into the CNS, although we cannot deny the possibility that SR-PSOX-CXCL16 is also involved in accumulation, activation, and/or proliferation of MOG_{35-55} peptide-specific, Th1-poralized, activated CD4 T cells in the CNS.

Previous reports clearly indicated the essential role of MCP-1/CCL2 in adoptive transfer EAE (11, 13), and MCP-1/CCL2-attracted monocyte/macrophage may induce traffic of activated T cell into the CNS by the function of SR-PSOX/CXCL16. SR-PSOX/CXCL16 that induces chemotaxis and accumulation of MOG_{35-55}-specific T cells expressing CXCR6 (Figs. 5 and 6*D*) might be expressed on infiltrated monocyte/macrophage into the CNS. Actually, SR-PSOX/CXCL16 was shown to be expressed on activated macrophages (14) and was suggested to be expressed in infiltrated cells into the CNS of EAE mice (Fig. 6*B*). However, we could not detect significant expression of SR-PSOX/CXCL16 in the spinal cords before immunization by in situ hybridization (Fig. 6, *B* and *C*). These data suggest that adoptively transferred MOG-reactive T cells infiltrate into the spinal cords of unimmunized mice without using CXCR6 and SR-PSOX/CXCL16, although administration of anti-SR-PSOX/CXCL16 mAb into the recipient mice inhibits onset of transfer EAE, probably through inhibiting direct infiltration of the transferred MOG-reactive T cells into spinal cord. Low level expression of SR-PSOX/CXCL16, which could be slightly detected by RT-PCR (Fig. 6*D*) but not by in situ hybridization (Fig. 6*C*) in the spinal cords of unimmunized mice,

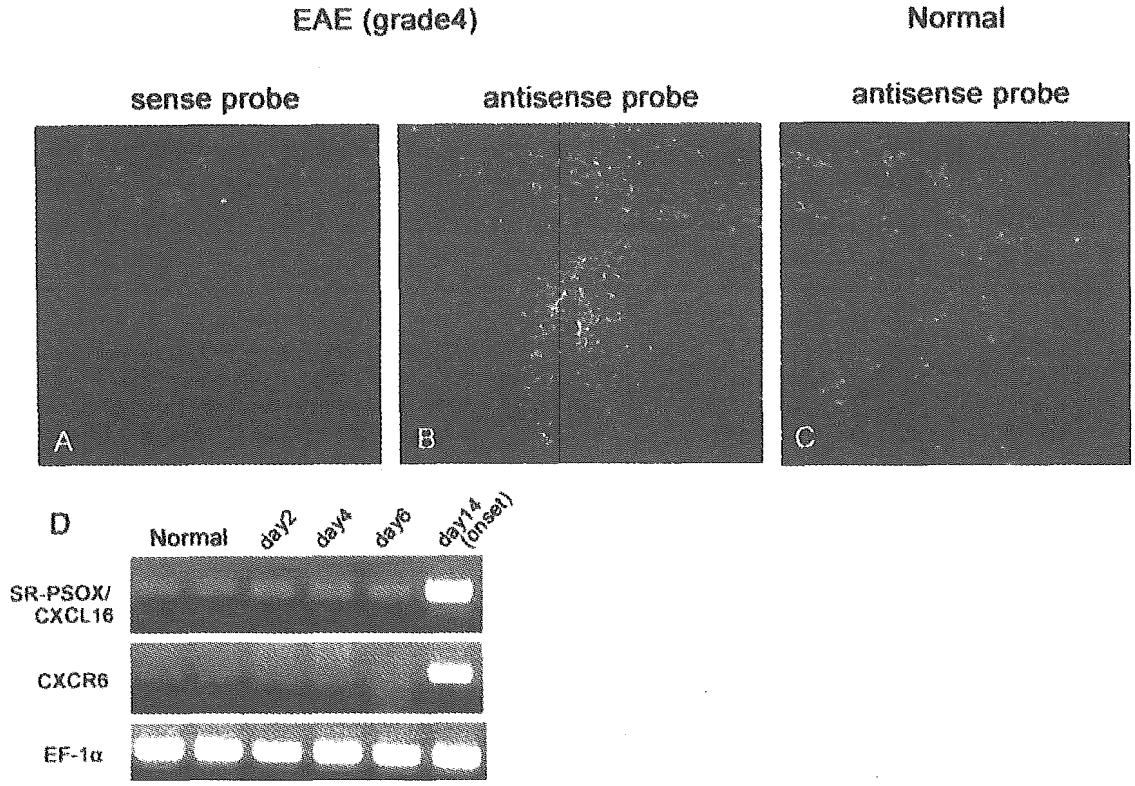

FIGURE 6. Expression of SR-PSOX/CXCL16 in the CNS of mice with EAE. *A–C*, Expression of SR-PSOX/CXCL16 mRNA was analyzed in spinal cord of EAE-induced mice 14 days after immunization or of control mice without immunization (normal) by in situ hybridization. *D*, mRNA of SR-PSOX/CXCL16 and CXCR6/Bonzo in spinal cord from EAE mice were measured by RT-PCR 2, 4, 6, and 14 days after immunization. Expression of elongation factor 1-α (EF1-α) mRNA was also measured as control.

might induce traffic of the transferred MOG-reactive T cells, although we cannot deny the possibility that SR-PSOX/CXCL16 does not play a role in infiltration of the adoptively transferred T cells into spinal cord.

MIP-1α/CCL3 and IP-10/CXCL10, which possess similar functions to SR-PSOX/CXCL16 against activated T cells, might be also involved in activated T cell traffic into the CNS in EAE (7, 8). However, two contradictory results were reported on the function of IP-10/CXCL10 for EAE, which indicated that administration of neutralizing Abs decreased or increased clinical disease incidence and severity, as well as infiltration of mononuclear cells into the CNS in transfer EAE, while anti-IP-10/CXCL10 did not show any effects on acute EAE (8, 10). SR-PSOX/CXCL16 and MIP-1α/CCL3 may coordinately and/or complementarily function in EAE together with or without IP-10/CXCL10 by inducing chemotaxis of activated Th1 T cells into the CNS, and/or accumulation of the activated T cells in the CNS.

Our findings in this report open the possibility that mAb-induced in vivo inhibition of biological activities of SR-PSOX/CXCL16, such as generation of Ag-specific T cells in primary immune response in acute EAE and traffic of Th1-poralized activated T cells into the CNS in transfer EAE, may be useful for clinical therapy of autoimmune diseases including MS.

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Peroxisome proliferator-activated receptor α ligands activate transcription of lectin-like oxidized low density lipoprotein receptor-1 gene through GC box motif☆

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Abstract

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is a receptor for oxidized LDL. Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors regulating transcription of various genes. We examined effects of PPAR ligands on LOX-1 expression and their transcriptional regulation in vascular endothelial cells. PPARα-specific ligands, such as fenofibrate and WY-14643, but not PPARγ-specific ligands induced LOX-1 expression. Reduced expression of PPARα by antisense oligonucleotides directed to PPARα blocked fenofibrate-induced LOX-1 expression. Luciferase reporter gene assays with deletion and point mutations in the LOX-1 promoter revealed that transcriptional activity of LOX-1 gene by fenofibrate was localized in the −114/−106 GC box. Electrophoretic mobility shift assays with the radiolabeled GC box sequence showed inducible bands by PPARα ligands, which is competitively suppressed by unlabeled GC box motif and by an antibody to PPARα. In conclusion, PPARα appears to be one of the key regulators that induce LOX-1 expression, utilizing the GC box as a promoter.

Keywords: Lectin-like oxidized low-density lipoprotein receptor-1; Peroxisome proliferators-activated receptors α; Vascular endothelial cells; GC box like motif; Oxidized LDL

Receptor-mediated endocytosis of oxidized LDL (Ox-LDL) may be crucial in the actions of Ox-LDL on vascular cells in atherogenesis. Among several different classes of receptors for atherogenic Ox-LDL, lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is a 40–50 kDa type II membrane glycoprotein with a C-type lectin-like extracellular domain and a short cytoplasmic tail [1]. Expression of LOX-1 can be found in vascular endothelial cells, in addition to macrophages, and activated vascular smooth muscle cells [2–4]. More importantly, LOX-1 expression is not constitutive, but drastically inducible by proinflammatory cytokines, such as tumor necrosis factor α (TNFα) [3,5] and transforming growth factor β (TGFβ) [6], and fluid shear stress [7]. Moreover, LOX-1 expression in vivo is highly upregulated in endothelial cells covering early atherosclerotic lesions, as well as macrophages and smooth muscle cells accumulated in the intima of advanced atherosclerotic lesions [8]. However, transcriptional regulatory mechanisms of the LOX-1 gene have not yet been elucidated.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily, which can activate the transcription of various genes by

☆ *Abbreviations:* LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; Ox-LDL, oxidized low-density lipoprotein; PPAR, peroxisome proliferators-activated receptor; PMA, phorbol 12-myristate 13-acetate; BAEC, bovine aortic endothelial cell.

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heterodimer formation with the retinoid X receptor (RXR) in ligand-activated manners, and play key roles in lipid and carbohydrate metabolism [9]. PPARα appears to play a key role in lipid metabolism to regulate the gene expression involved in lipid metabolism [10]. PPARs are also expressed by vascular endothelial and smooth muscle cells [11–14] as well as macrophages [15].

PPARγ ligands can stimulate macrophage differentiation and induce expression of CD36, a receptor for Ox-LDL [15]. PPARα ligands, including fibrates [16], can suppress expression of proinflammatory genes, such as VCAM-1 [17] and IL-6 [14], in vascular endothelial and smooth muscle cells. By contrast, recent studies showed oxidized phospholipids could directly activate PPARα [18,19] to induce MCP-1 and IL-8 in vascular endothelial cells [19]. To further explore the direct action of PPARs on vascular cells, we sought to determine whether PPARα ligands can modulate expression of LOX-1 which is endocytic receptor for Ox-LDL in cultured vascular endothelial cells. We also explored transcriptional regulatory mechanisms involved in this process.

Materials and methods

Cell culture and reagents. DMEM was obtained from Nissui and fetal bovine serum (FBS) was from Irvine Scientific. WY14643 was purchased from Calbiochem–Novabiochem. Fenofibrate and 15-deoxy-12,14-prostaglandin J_2 was purchased from Sigma. Troglitazone was kindly provided by Sankyo. Bovine aortic endothelial cells (BAECs) were isolated by scraping the inner surface of bovine aortas with a razor blade and cultured in DMEM containing 10% (vol/vol) FBS.

Immunoblot analysis. Cells were washed with PBS and lysed in a buffer containing 50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.01% bromophenol blue. After heated at 98 °C for 5 min, equal protein concentrations of the cell lysates were subjected to SDS–polyacrylamide (10%) gel electrophoresis and transferred onto nitrocellulose membranes (Hybond-N$^+$, Amersham) by electroblotting. After preincubation with blocking reagent (PBS containing 0.1% Tween 20 and 5% nonfat dried milk) for 2 h at room temperature, blotted membranes were incubated with an anti-bovine LOX-1 monoclonal antibody for 2 h at room temperature, followed by washing twice with blocking reagent. Membranes were then incubated with a horseradish peroxidase-conjugated anti-mouse IgG (Amersham) for 1 h at room temperature, washed twice in PBS containing 0.04% Tween 20, and visualized by ECL Western blotting detection reagents (Amersham Biosciences).

Northern blot analysis. Total cellular RNA was isolated by Trizol reagent (Invitrogen). Total cellular RNA (15 μg) was subjected to electrophoresis through 1% agarose gel containing formaldehyde and transferred onto nitrocellulose membranes (Pro-Tran, Schleicher & Schuell). Membranes were hybridized with an *Xho*I fragment of bovine LOX-1 cDNA which had been labeled with [α-^{32}P]dCTP (DuPont-New England Nuclear) using random hexanucleotide primers (DNA labeling kit, Amersham Biosciences).

Cellular uptake of DiI-labeled Ox-LDL. LDL (density: 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation of human plasma. Oxidative modification of LDL was carried out with cupric ion in vitro as previously described [20]. Oxidation was monitored by measuring the amount of thiobarbituric acid-reactive substances (TBARS). Our Ox-LDL contained approximately 10 nmol malondialdehyde equivalent/mg protein. Agarose gel electrophoresis showed increased electrophoretic mobility and minimal aggregation of Ox-LDL particles. Labeling of LDL with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI, Molecular Probes) was performed as previously described [21]. To examine cellular uptake of Ox-LDL, BAECs were incubated with DiI-labeled Ox-LDL (5 μg/ml) with 500 μg/ml (the 100-fold excess amount) of unlabeled acetylated LDL (Ac-LDL) in DMEM/10% FBS for additional 2 h after treatment of the indicated reagents for 12 h, and washed three times with the cell culture medium. Fluorescence microscopy was performed to detect DiI-Ox-LDL accumulated in cytoplasm. To quantify the fluorescence intensity, DiI was extracted from the cells by isopropanol, and the fluorescence intensity was measured in Fluoroscan II (Flow Laboratories). Acetylation of LDL was carried out using acetic anhydride as previously described [1].

Antisense oligonucleotides directed to PPARα. Antisense, sense, missense, and scrambled phosphorothionate oligonucleotides (ODNs) directed to 5′-coding sequence of the bovine PPARα mRNA were designed and manufactured by Fasmac. The sequences of ODNs were as follows: antisense; 5′-GGGCTTTCCGTGTCCACCAT, sense; 5′-ATGGTGGACACGGAAAGCCC, missense; 5′-GGCCTTGCCG TATCCATCAT, and scramble; 5′-TACCACCTGTGCCTTTCG GG. Transfection into BAECs was performed at the concentration of 1 μM of sODNs with lipofectamine 2000 (Invitrogen). After transfected cells were incubated with or without 100 μM fenofibrate for 12 h, the whole cell lysates were subjected to immunoblotting with an anti-LOX-1 monoclonal antibody.

RT-PCR for PPARα mRNA expression. Total RNA (1 μg) extracted from BAECs was reverse-transcribed with oligo(dT) and Superscript II (Invitrogen). The reverse-transcribed material (3 μl) was amplified with Ex-taq (Takara) by use of pairs of primers specific to bovine PPARα (forward primer: GGATCAGATGGCTCCGTTATT ACAG, reverse primer: GCTCCAGTGCATTGAACTTCATTG) and bovine glyceraldehyde-phosphate-dehydrogenase (forward primer: TCTGGCAAAGTGGACATCG, reverse primer: GGTCATAAGTC CCTCCACGAT). PCR products were 925 and 456 bp, for PPARα and GAPDH, respectively. For PCR, 30 cycles were used at 94 °C for 40 s, 55 °C for 1 min, and 72 °C for 1 min. PCR amplified products were visualized on 1.0% agarose gel electrophoresis by ethidium bromide staining.

Luciferase reporter gene plasmid constructs. Promoter regions of human LOX-1 gene from −3141 to +31, from −2423 to +31, and −167 to +31 were amplified from genomic DNA isolated from Jurkat cells by PCR and subcloned into PGL3-Basic (Promega), and were designated PGL3 −3141/+31, PGL3 −2433/+31, and PGL3 −167/+31, respectively. Reporter plasmids containing promoter regions from −1662 to +31 and from −1136 to +31 were generated by self-ligation of PGL3 −2423/+31 after digestion with *Sma*I and *Eco*RV. Plasmid containing from −527 to +31 was generated by self-ligation of PGL3 −2423/+31 after digestion with *Sma*I and *Spe*I followed by blunting. A point mutation with transversion of G–T at −107 in GC box related sequence located between −114 and −106 was generated by LA PCR in vitro mutagenesis kit (Takara).

Promoter–reporter gene assay. Confluent BAECs cultured in 24-well plates were transiently transfected with 2 μg of a luciferase reporter gene construct containing human LOX-1 5′ promoter region in 100 μl of Optimem (Invitrogen) and 0.2 μg pRL-TK plasmid (Promega) simultaneously as an internal transfection control using lipofectamine 2000 (Invitrogen). Luciferase activities were determined by dual-luciferase assay kit (Promega), and transfection efficiencies were normalized by *Renilla* luciferase activities.

Nuclear protein extraction and electrophoretic mobility shift assay (EMSA). Nuclear proteins were extracted, as previously described [22], from BAECs. Binding reactions were carried out in a total volume of 10 μl containing 10 μg of nuclear extracts, 1 μg of salmon testis DNA (Sigma), and ^{32}P-labeled oligonucleotide probes at room temperature for 30 min. Sequences of the used oligonucleotides were as

follows (only the one strand is shown). LOX1-GC; 5′-GAATTTGC GTCAGCGAACTTCTT, ACO-DR1; 5′-GAACGTGACCTTTGTC CTGGT which is corresponding to the PPRE for the rat acyl-CoA oxidase gene. In some experiments, nuclear extracts were preincubated with excess amount of unlabeled oligonucleotides, 1 μg of goat control IgG, or 1 μg of a goat anti-PPARα polyclonal antibody (Santa Cruz Biotechnology) at room temperature for 30 min. These mixtures were subjected to electrophoresis through 5% (wt/vol) polyacrylamide gels containing 10% glycerol. The dried gels were analyzed by Fuji Bio-image Analyzer BAS2400 (Fuji Photo Film).

Statistical analysis. Statistical significance in differences between groups was determined by one factor analysis of variance with Fisher PLSD as post hoc test, and statistical significance between two groups was determined by Student's *t* test. A value of $p < 0.05$ was considered significant.

Results

LOX-1 expression is upregulated by PPARα ligands but not by PPARγ ligands in cultured bovine aortic endothelial cells

After confluent monolayers of bovine aortic endothelial cells (BAECs) were treated with or without various PPARα ligands, such as fenofibrate and WY14643, as well as PPARγ ligands, such as troglitazone and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), for 12 h, total cell lysates and total cellular RNA were isolated and subjected to immunoblot and Northern blot analyses, respectively. As shown in Fig. 1A, the bar graph, indicating semi-quantification of LOX-1 protein expression, shows that PPARα ligands, such as fenofibrate (100 μM) or WY14643 (250 μM), prominently induced LOX-1 protein expression. In contrast, PPARγ ligands, such as troglitazone (15 μM) or 15d-PGJ_2 (3 μM), did not significantly affect LOX-1 protein expression. Fig. 1B demonstrates that LOX-1 mRNA was remarkably induced by fenofibrate and WY14643 (PPARα ligands), but not by 15d-PGJ_2 or troglitazone (PPARγ ligands), as shown by Northern blotting. Treatment with various concentrations of fenofibrate (0–200 μM) showed that LOX-1 protein expression was upregulated in a dose-dependent manner (Fig. 2A). Time-course experiments showed that increased LOX-1 protein expression was found as early as 3 h after the addition of fenofibrate, peaked after 24 h, and sustained for 48 h (Fig. 2B).

PPARα ligands increased the uptake of DiI-labeled oxidized LDL which was not inhibited by excess amounts of unlabeled acetylated LDL

To determine whether upregulated expression of LOX-1 by PPARα ligands is correlated with enhanced uptake of oxidized LDL (Ox-LDL), amounts of DiI-labeled Ox-LDL internalized into BAECs were measured. Because LOX-1 can take up Ox-LDL but not Ac-LDL, we measured the amounts of DiI-labeled Ox-LDL taken

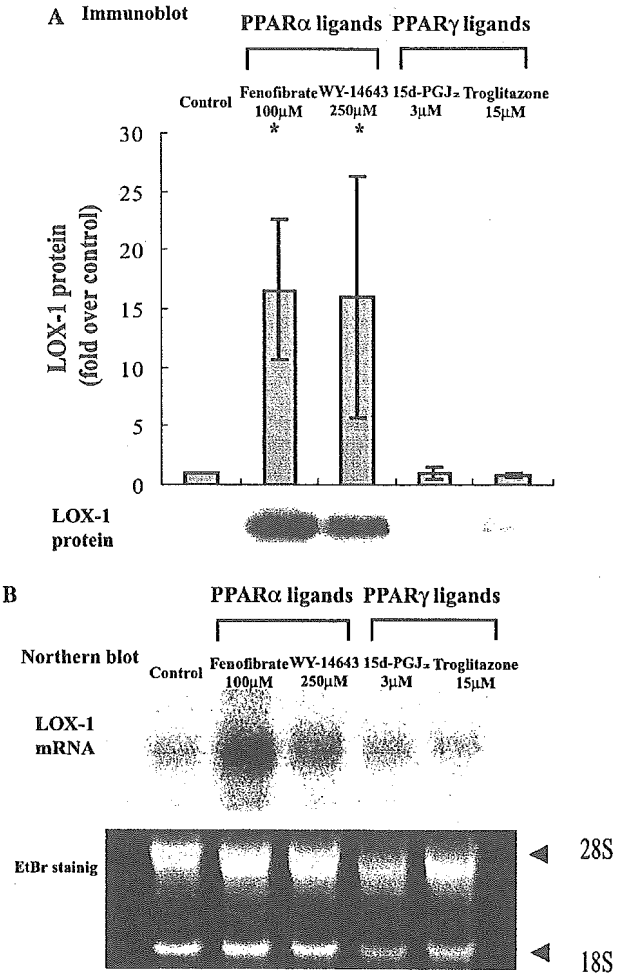

Fig. 1. Induction of LOX-1 expression by PPARα ligands in BAECs. (A) Immunoblot analyses for LOX-1 in BAECs treated with various PPAR ligands. Cells were treated with or without (control) the indicated concentrations of PPAR ligands for 16 h and subjected to immunoblot analyses. A bar graph indicates semi-quantification analysis from three independent experiments. Mean values and the standard deviations are indicated. LOX-1 protein induced by fenofibrate or WY14643 was significantly increased, compared to control ($p < 0.05$). There are no significant differences between that of fenofibrate and WY 14643. A representative figure of immunoblotting is also shown. (B) Northern blot analyses for LOX-1 mRNA in BAECs treated with various PPAR ligands. BAECs were treated with or without (control) the indicated concentrations of PPAR ligands for 16 h and subjected to Northern blot analyses. Each lane contained 15 μg of total RNA. A representative figure is shown from three independent experiments. Lower panel shows ethidium bromide staining of the RNA gel.

up by Ox-LDL- specific pathways that cannot be inhibited by excess amounts of unlabeled Ac-LDL. After treatment with or without PPAR ligands for 12 h, BAECs were incubated with DiI-labeled Ox-LDL in combination with the 100-fold excess amounts of unlabeled Ac-LDL for additional 2 h. PPARα ligands, but not a PPARγ ligand troglitazone, increased the internalization of DiI-Ox-LDL, as shown in Fig. 3A.

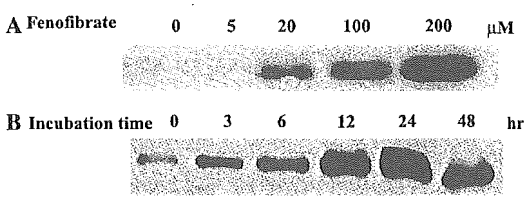

Fig. 2. Dose–response relationship (A) and time-course (B) of LOX-1 protein expression induced by a PPARα ligand, fenofibrate. (A) BAECs were treated with the indicated concentrations of fenofibrate for 12 h, and subjected to immunoblot analyses. (B) BAECs were treated with 100 μM of fenofibrate for the indicated time periods and subjected to immunoblot analyses.

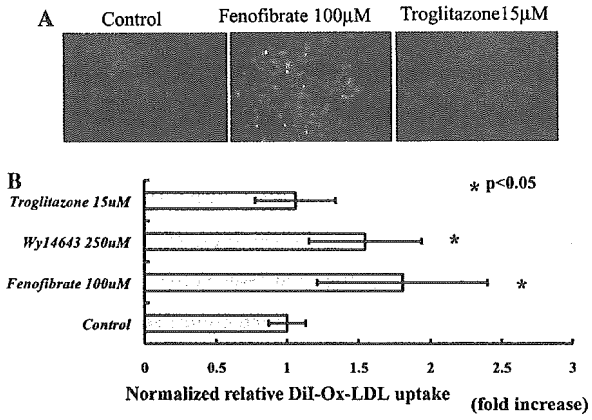

Fig. 3. PPARα ligands increased the specific uptake of Ox-LDL which is distinct from acetylated LDL uptake. After treatment with the indicated reagents for 12 h, BAECs were incubated with 5 μg/ml of DiI-labeled Ox-LDL in combination with 500 μg/ml of unlabeled Ac-LDL for an additional 2 h. (A) Fluorescence microscopic pictures are shown. (B) Quantitative analyses of DiI-labeled Ox-LDL uptake in BAECs treated with the indicated reagents. Columns indicate the mean of relative fluorescence intensities normalized against cellular protein concentrations from six wells. Bars represent standard deviations.

Quantification of the DiI-Ox-LDL uptake in BAECs showed that PPARα ligands enhanced Ox-LDL specific uptake by approximately 2-fold, but troglitazone did not (Fig. 3B). These results demonstrated that increases in LOX-1 expression by PPARα ligands were associated with enhanced Ox-LDL-specific uptake in BAECs.

Inhibition of PPARα expression by antisense oligonucleotides suppressed fenofibrate- induced LOX-1 expression

To confirm that fenofibrate-induced LOX-1 expression depends upon PPARα, effects of antisense ODNs against PPARα were examined. Transfection of antisense ODNs directed to PPARα significantly suppressed PPARα mRNA expression as expected. In contrast, neither sense, missense nor scrambled ODNs suppressed PPARα expression. PPARα/GAPDH mRNA ratios were as follows; antisense: 0.13 ± 0.03, sense: 0.55 ± 0.06 missense: 0.27 ± 0.10, and scrambled: 0.43 ± 0.07 ($p < 0.05$: antisense vs. sense, missense or scrambled; Fig. 4A). As shown in Fig. 4B, reduced expression of PPARα resulted in suppressed LOX-1 expression induced by fenofibrate. Transfection of sense, missense, or scrambled, ODNs against PPARα did not significantly reduce fenofibrate-induced LOX-1 expression (antisense: 2.2 ± 0.6-fold, sense: 6.7 ± 0.7-fold missense: 6.2 ± 1.3-fold, and scrambled: 6.1 ± 1.5-fold increases compared with untreated control, $p < 0.05$: antisense vs. sense, missense or scrambled; Fig. 4B).

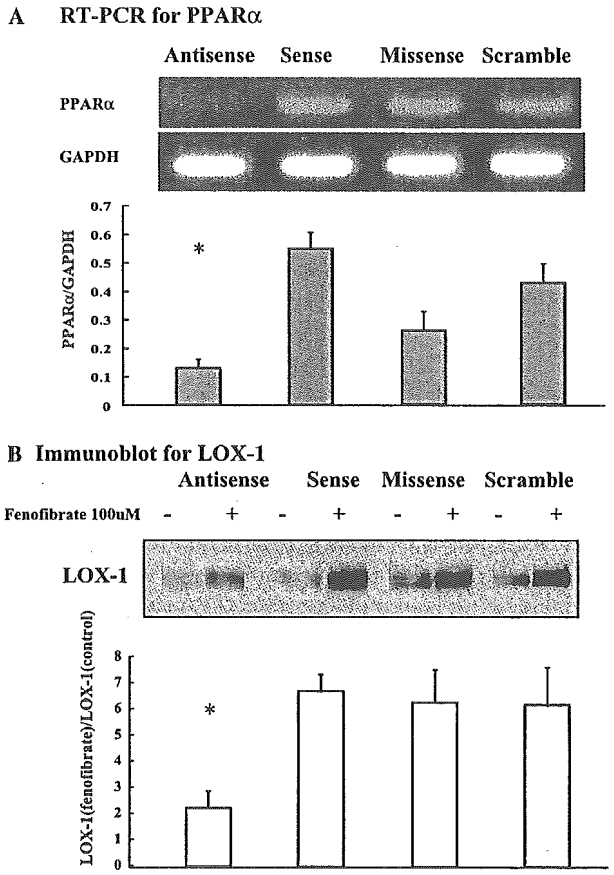

Fig. 4. Effects of antisense oligonucleotides (sODN) for PPAR α on fenofibrate-induced expression of LOX-1 BAECs were transfected with 10 μM of the indicated sODN by use of Lipofectamine 2000. After 24 h incubation, these cells were incubated with or without fenofibrate for additional 16 h, and then subjected to RT-PCR for PPARα (A) and immunoblotting for LOX-1 (B). (A) Antisense sODN for PPARα significantly suppressed PPAR α mRNA expression. Total RNA isolated from BAECs was subjected to RT-PCR. Upper panel shows the representative figure. Lower panel shows quantification of PPARα/GAPDH mRNA ratio. (*$p < 0.05$ compared with sense, missense, or scrambled sODN). (B) Increased expression of LOX-1 was blocked by antisense sODN. Upper panel shows a representative figure of immunoblotting for LOX-1. Lower panel indicates a bar graph showing semi-quantification of LOX-1 expression by fenofibrate. Mean values and the standard deviations calculated from 5-wells are indicated. (*$p < 0.05$ compared with sense, missense, or scrambled sODN).

GC box motif at −114/−106 region of the LOX-1 gene promoter is responsible for PPARα-induced transcription of LOX-1 gene

We have further analyzed transcriptional regulation of LOX-1 gene by PPARα. Schematic illustration of the luciferase-reporter constructs used in this study, containing various lengths of 5′ flanking region of human LOX-1 gene with or without mutated GC box, is shown in Fig. 5A. All these constructs contained TPA-responsive element (TRE) at −60/−53. At 24 h after transfection with the indicated promoter–reporter gene constructs, BAECs were incubated with or without fenofibrate (100 μM) or WY14643 (250 μM) for additional 12 h, and then luciferase activities were measured. promoter–reporter gene constructs containing −167/+31 region, including GC box, showed significant increases, which is comparable with those containing −3141/+31 or −2423/+31, in the luciferase activity by fenofibrate or WY14643; however, a point mutation in the GC box (PGL3-p2423M and PGL3-p167M) did not show statistically significant increases by fenofibrate or WY14643 (asterisks; Fig. 5B). In contrast, both promoter–reporter constructs containing wild-type and mutated GC box motif (PGL3-p167 and PGL3-167M) responded well to phorbol 12-myristate 13-acetate (PMA) stimulation (Fig. 5B), indicating that this GC box is not necessary for PMA-induced LOX-1 gene transcription but TPA-responsive elements (TRE) at −60/−53 might be involved. These results thus indicate that the GC box motif located in −167/+31 region of the LOX-1 gene is a specific *cis*-element for PPARα-induced transcription of LOX-1 gene.

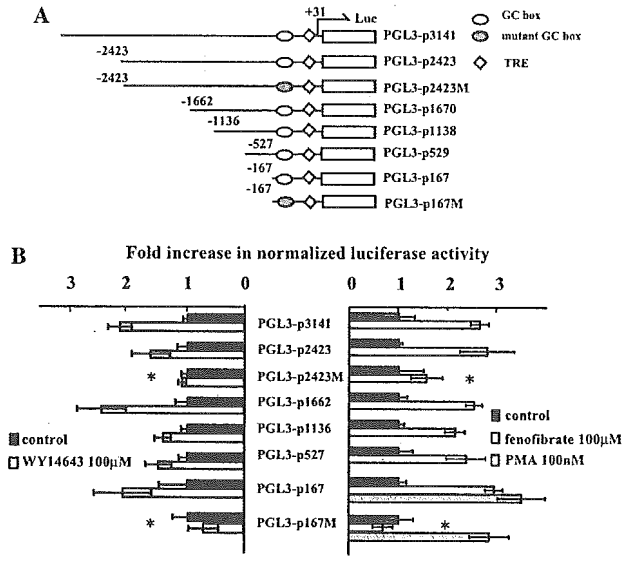

Fig. 5. Identification of a *cis*-acting element responsible for LOX-1 gene transcription by PPARα ligands. (A) Scheme of LOX-1 promoter-luciferase gene constructs containing various lengths of 5′ flanking region of the LOX-1 gene with or without the mutated GC box. (B) BAECs were transfected with the indicated promoter–reporter gene constructs. After 24 h incubation, these cells were incubated with or without fenofibrate (100 μM), WY14643 (250 μM), or phorbol 12-myristate 13-acetate (PMA; 100 nM) for additional 12 h, and then luciferase activities were measured. Fold increases compared to mean values of the controls after normalization by an internal transfection control (pRL-TK) are indicated. Data are means ± standard deviations from five samples for each experimental condition. Asterisks (*) indicate that significant increases in luciferase activities by fenofibrate or WY14643 were abrogated.

Activated PPARα binds to the GC box motif in the promoter region of LOX-1 gene.

To determine whether activated PPARα by fenofibrate in nuclei can bind to the GC box motif in the promoter region of human LOX-1 gene, an electrophoretic mobility shift assay (EMSA) was performed using nuclear extract from BAECs incubated with or without fenofibrate, using the GC box motif oligonucleotides (LOX1-GC) or the oligonucleotides corresponding to PPRE for acyl-CoA oxidase gene (ACO-DR1) as a probe. As shown in Fig. 6, nucleoprotein complex bound by LOX1-GC was induced by 50 μM fenofibrate, which was inhibited by 100-fold excess amounts of unlabeled LOX1-GC or unlabeled ACO-DR1 that has been shown to bind activated PPARα [23,24]. Coincubation with an anti-PPARα antibody also abolished the complex formation, although control IgG did not. Furthermore, nucleoprotein complex bound by ACO-DR1 showed almost the same electrophoretic mobility (Fig. 6). These results indicate that activated PPARα can bind to the GC box motif in the promoter region of human LOX-1 gene and that its binding site for the GC box in the LOX-1 gene overlaps with that for the PPRE in the acyl-CoA oxidase gene.

Discussion

Previous studies have suggested that endothelial activation elicited by Ox-LDL and its lipid constituents may be involved in initiation and progression of atherosclerosis [25–27]. Ox-LDL uptake mediated by LOX-1 has been shown to induce cellular oxidative stress, activation of the proinflammatory transcription factor NF-κB [28], production of matrix metalloproteinases [29], expression of monocyte chemotactic protein-1 (MCP-1) [30], and cell apoptosis [31], which may potentially enhance atherosclerotic progression and stimulate rupture of atheromatous plaques.

On the other hand, PPARα and γ are also expressed by vascular endothelial cells and smooth muscle cells [11–14,17]. Previous reports have shown that PPARα ligands can suppress expression of proinflammatory genes, such as VCAM-1, IL-6, and COX-2, in vascular

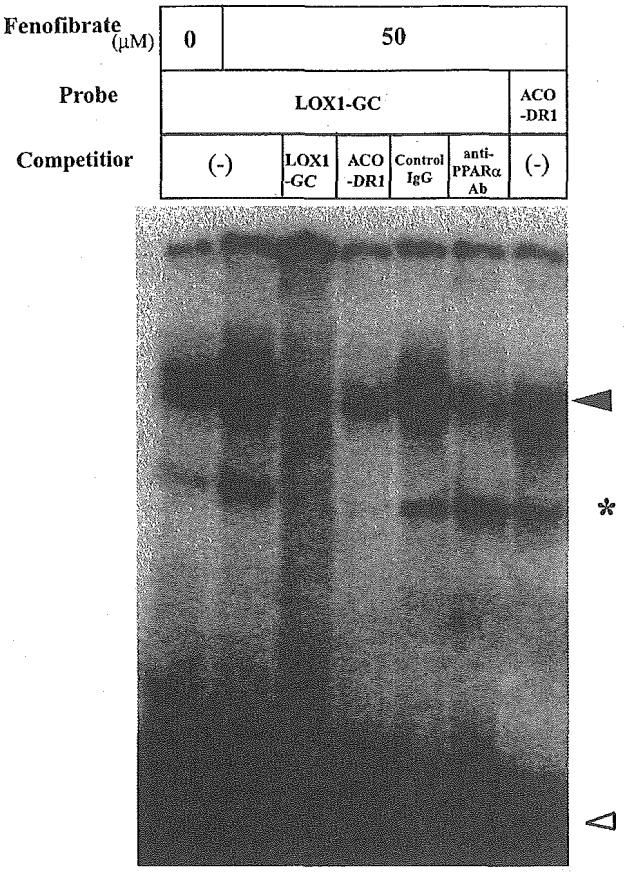

Fig. 6. Activated PPARα binds to the GC box in the LOX-1 promoter After treated with or without 50 or 100 μM of fenofibrate for 24 h, nuclear protein was extracted from BAECs and then subjected to EMSA. Double stranded ^{32}P-labeled nucleotides corresponding to GC box motif in LOX-1 promoter (LOX1-GC) or PPRE in rat acyl-CoA oxidase promoter (ACO-DR1) were used as probes as indicated. An antibody directed to PPARα, control IgG, and unlabeled competitor oligonucleotides were also added in the binding reaction as indicated. A closed arrowhead indicates the band induced by fenofibrate treatment and abolished by coincubation with unlabeled competitors or with anti-PPARα antibodies. An open arrowhead indicates free probe. An asterisk indicated a fenofibrate-induced shift band which was not suppressed by an anti-PPARα antibody.

endothelial or smooth muscle cells [14,17,32]. PPARγ ligands increase PAI-1 expression in vascular endothelial cells [11] and induce CD36 expression in macrophages [15]. Target genes of PPARα are relatively confined to a group of genes which are involved in the metabolism of fatty acids [33–36] and lipoproteins [37,38]. In addition to these target genes, our study has revealed, for the first time, that LOX-1, a scavenger receptor for atherogenic Ox-LDL and other physiological ligands [39–41], is also a target of PPARα in vascular endothelial cells. Moreover, recent studies showed oxidized phospholipids, constitutes of Ox-LDL, directly activated PPARα to induce expression of MCP-1 and IL-8, which was completely abolished in endothelial cells from PPARα null mice [19]. LOX-1 expression is upregulated by Ox-LDL both in endothelial cells [42] and vascular smooth muscle cells [31]. In addition, suppression of LOX-1 inhibits Ox-LDL-induced MCP-1 expression in endothelial cells [30]. Thus, PPARα-induced LOX-1 expression might be involved in Ox-LDL-induced MCP-1 expression in atherogenesis.

In general, PPARs can stimulate gene transcription by heterodimer complex formation with RXR and its interaction with the *cis*-acting element, PPRE [43–45]. Although the consensus nucleotide sequence for PPRE was undetectable in the 5′ flanking region of the LOX-1 gene, we have identified, for the first time, the GC box motif as a responsible element for PPARα-induced gene transcription. However, in EMSA (Fig. 6) another inducible band by fenofibrate treatment was also visible below the PPAR band as indicated by the asterisk. This band was not reduced by addition of an anti-PPARα antibody; therefore, this band might correspond to another fenofibrate-inducible nuclear protein which can be bound to DR-1 sequence and the GC box motif. In addition, this report is the first to demonstrate transcriptional regulation of the LOX-1 gene. PPARα have been reported to regulate gene transcription by interacting with other transcription factors or cofactors, such as CBP [46], PGC-1 [47], and SRC-1 [48]. Therefore, transcriptional activation by PPARα via the GC box motif may be mediated by its interaction with cofactors other than RXR. This point needs to be further explored in the future.

In summary, the present report provides evidence, for the first time, that endothelial LOX-1 expression can be upregulated by PPARα via the GC box motif in the promoter region. Further studies would elucidate the pathophysiological relevance of PPARα ligand-induced endothelial LOX-1 expression in atherogenesis and other pathophysiological settings in vivo.

Acknowledgments

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REVIEW

Biochemical markers of myocyte injury in heart failure

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This review discusses the role of biochemical markers of myocyte injury in patients with chronic congestive heart failure. Heart specific assays have been developed for the measurement of cardiac troponin T (cTnT), cardiac troponin I (cTnI), heart type fatty acid binding protein (H-FABP), and myosin light chain 1 (MLC-1). Concentrations of these biochemical markers increase in the absence of ischaemic events in the subset of patients with heart failure whose long term outcomes are most adverse. The markers are easy to measure serially and it is therefore easy to follow patients without inter-observer variability. The serial clinical use of these markers, separately or in combination, will sharpen our understanding of the state of heart failure.

Chronic congestive heart failure is associated with a dismal long term prognosis and remains a major worldwide health concern. While various management strategies have become available, clinical tools to stage chronic heart failure remain few. New York Heart Association (NYHA) functional classification, along with several tests, including chest *x* ray, echocardiogram, myocardial scintigraphy, cardiopulmonary exercise, and haemodynamic measurements are useful to estimate the degree of heart failure, though are subject to inter-observer variations in interpretation.[1 2]

The loss of cardiac function in patients with chronic heart failure may be caused by ventricular remodelling, a process by which ventricular size, shape, and function are altered by mechanical, neurohormonal, and genetic factors. Ventricular remodelling consists of myocyte hypertrophy and slippage, loss of myocytes, decrease in myofibril content, and myocardial interstitial fibrosis.[3 4] Serial measurements of reliable biochemical markers of myocyte injury in patients with heart failure would be helpful to monitor their long term progress, without inter-observer variability. This review discusses our understanding, and the significance, of current biochemical markers detected in patients with chronic heart failure.

BIOCHEMICAL MARKERS OF MYOCYTE INJURY

Cardiac enzymes have long been used as front-line diagnostic tools in the detection of myocardial injury caused by myocardial ischaemia.[5] However, the most commonly used enzymes, including creatine kinase (CK) and its myocardial fraction CK myocardial band (MB), aspartate aminotransferase, and lactate dehydrogenase, are limited in their ability to detect myocardial injury by short diagnostic windows, limited sensitivities, and lack of specificity because of their presence in skeletal muscle. Furthermore, studies performed as long as two decades ago found no correlation between serum concentrations of CK or CK-MB and heart failure.[6] Myoglobin, an 18 kD cytosolic protein, also lacks specificity because its release from skeletal muscle cannot be distinguished from its release from heart muscle.[7]

These limitations, as well as the known unique amino acid sequence of myofibrillar cardiac proteins, prompted the development of monoclonal antibodies for the detection of cardiac troponins by immunoassay. The subunits I, T, and C of the troponin complex on the actin filament regulate the force and velocity of muscle contraction. The 37 kD T subunit is responsible for binding the troponin complex to tropomyosin. The first generation of cTnT assays were flawed by spuriously increased values in patients with severe skeletal muscle or renal disorders,[8 9] perhaps from cross-reactivity of the cTnT assay with skeletal muscle troponin T, or the expression of cTnT by skeletal muscles during regenerative processes, particularly in patients with neuromuscular disorders and nephropathies. However, the latest cTnT assay is a sensitive and specific marker of myocyte injury, even in the presence of these disorders.[10 11]

cTnI (21 kD) prevents contraction in the absence of calcium by inhibiting the adenosine triphosphatase activity of the actin–myosin interaction. cTnI is highly cardiac specific because of the dissimilarity of a 31 amino acids sequence on the N-terminus compared to that of skeletal troponins; it is also absent during human skeletal muscle regeneration.[12] cTnT and cTnI are highly sensitive and specific markers of myocardial injury in acute coronary syndromes. In addition, increased serum concentrations of these markers have been associated with adverse short and long term outcomes in patients with unstable angina or acute myocardial infarction.[13 14] Moreover, the background concentration of cTnT and cTnI is very low while the background level of CK, aspartate aminotransferase, and lactate dehydrogenase is significant even in normal conditions. cTnT and cTnI,

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Abbreviations: BNP, brain natriuretic peptide; cTnI, cardiac troponin I; cTnT, cardiac troponin T; CK, creatine kinase; DCM, dilated cardiomyopathy; H-FABP, heart type fatty acid binding protein; MB, myocardial band; MLC-1, myosin light chain 1; NYHA, New York Heart Association

therefore, have replaced CK-MB as the standard marker in acute coronary syndromes, and a new definition of acute myocardial infarction has been developed, based on increases in cardiac troponins in the blood.[15 16]

The mechanisms of release and clearance of cTnT and cTnI are incompletely understood. Although both are structural proteins, it has been suggested that cytosolic pools of these proteins are released into the circulation after cell injury. The cytosolic pool for cTnT was estimated at 6–8%,[13] and that for soluble cTnI at 2.8%.[14] The release of cTnT in ischaemic myocardial injury may be because of transient leakage from the cytosolic component from loss of sarcolemmal integrity during reversible ischaemia,[17] or from its continuous release when ischaemic injury is irreversible.[18]

Other biochemical markers of myocyte injury have also been described. H-FABP, a 15 kD cytoplasmic protein involved in lipid homeostasis, is abundant in heart muscle.[19 20] It has recently been reported to detect early myocyte injury in patients with acute myocardial infarction.[21] Myosin is a structural protein of the sarcomere; a heart specific assay using monoclonal antibodies against MLC-1 has been previously described in patients with acute myocardial infarction.[22]

BIOCHEMICAL MARKERS OF MYOCYTE INJURY IN PATIENTS WITH HEART FAILURE

cTnT

Congestive heart failure is a clinical syndrome which may develop from a variety of diseases. Dilated cardiomyopathy (DCM) is a primary myocardial disorder of unknown aetiology characterised by ventricular dilatation and depressed myocardial contractility, which leads to chronic heart failure without apparent myocardial ischaemia. While ongoing myocyte injury has been documented by ^{111}indium anti-myosin antibody imaging in patients with DCM,[23] this technique requires radioisotopes and cannot be used to follow patients serially in the long term.

In an earlier study, persistently high serum concentrations of cTnT were observed over several years of follow up in approximately 30% of our DCM patients.[24 25] These patients had a significantly greater decrease in left ventricular ejection fraction and higher rates of long term adverse outcomes than patients without increased cTnT concentrations. It is particularly noteworthy that, in most patients, cTnT concentrations remained elevated after the patients were stabilised clinically by conventional therapy, free of dyspnoea, and without radiographic and auscultatory signs of pulmonary congestion. These observations indicate that cTnT is a marker of subclinical myocyte injury even when heart failure is compensated. In that study of patients with DCM, we chose 0.02 ng/ml as the upper normal limit of serum concentration, a relatively low value compared with patients with ischaemic heart disease.

Cardiomyopathic disorders are associated with predominantly systolic or diastolic dysfunction, or with both. In hypertrophic cardiomyopathy, which is initially associated with predominant diastolic dysfunction,[26] we recently reported increased concentrations of cTnT in 50% of patients during the non-dilated phase of the disease, when systolic function was preserved, and in the absence of ischaemia.[27] Some patients had increased cTnT concentrations persisting over several years of follow up, during which fractional shortening and intraventricular septum thickness decreased significantly. These observations indicate that cTnT is a marker of myocyte injury in patients with hypertrophic cardiomyopathy. In a univariate analysis, Dispenzieri and colleagues found cTnT, cTnI, septal thickness, left ventricular ejection fraction, urine M spike, age, and symptoms of congestive heart failure to be significant predictors of overall survival in patients with cardiac amyloidosis, while in multivariate analysis, the detection of cTnT was the most reliable predictor.[28]

Though we initially studied the significance of cTnT as a marker of myocyte injury in patients with heart failure and non-ischaemic disorders, ischaemic heart disease remains the predominant cause of chronic heart failure. We and others have reported the presence of increased cTnT concentrations in patients with heart failure, old myocardial infarctions, and without ongoing ischaemic events.[29 30] Ventricular remodelling after myocardial infarction may occur over several weeks or months, while other factors may also contribute to the progression of left ventricular dysfunction, including recurrent ischaemia with myocardial stunning, hibernation of the myocardium caused by a sustained reduction in myocardial blood flow, and the vascular and myocardial effects of endothelial dysfunction.[4]

While more studies of cTnT during cardiac remodelling after myocardial infarction should further promote its acceptance as a monitoring tool in patients with heart failure, the interpretation of results in patients with ischaemic heart disease is not without ambiguity. Since patients with heart failure after a healed myocardial infarction may have asymptomatic stenoses of one or more, large or small, coronary arteries, increased concentrations of markers of myocyte injury may be a manifestation of ischaemic myocardium in the territory of the stenotic artery. Therefore, patients with chronic heart failure after an old myocardial infarction may have to undergo serial coronary angiograms to clarify the mechanism of cTnT release.[31]

We have collected preliminary data in patients with old myocardial infarctions who have undergone coronary revascularisation, and had persistently elevated cTnT and progression of heart failure, in the absence of increased CK or ischaemic events (unpublished data). Although cTnT is usually elevated in patients with unstable angina or acute coronary syndrome,[32 33] the persistently high concentrations, measured for several months or years after myocardial infarction, in our patients with chronic heart failure could not be attributed to unstable angina. Further studies are needed to clarify the significance of persistently elevated cTnT in the process of chronic ventricular remodelling after myocardial infarction.

Valvar and congenital heart diseases are other major causes of chronic heart failure, which may progress despite successful surgical interventions.[34] We and others have found that some patients with valvar and congenital diseases also have elevated cTnT concentrations in the absence of cardiac ischaemia.[29 30 35] Our preliminary observations of persistently high cTnT concentrations in some patients after surgical repair may be useful for the post-operative monitoring of chronic myocyte injury (unpublished data).

cTnI

The first description of a biochemical marker of myocyte injury in patients with heart failure was offered in 1995 by Missov and colleagues, who reported increased cTnI concentrations in patients with NYHA class III and IV heart failure caused by DCM, or secondary to ischaemic disease.[36 37] Cardinale and associates reported that the elevation of cTnI in patients treated with high doses of chemotherapeutic agents for aggressive malignancies predicted the subsequent evolution of left ventricular function.[38] They concluded that cTnI is a sensitive and reliable marker of myocardial injury caused by high dose chemotherapy. Schultz and colleagues reported an exercise induced increase in cTnI concentrations in patients with heart failure, though the prognostic value of this finding should be further investigated.[39]