Propagermanium Reduces Atherosclerosis in Apolipoprotein E Knockout Mice via Inhibition of Macrophage Infiltration

Tomoya Yamashita, Seinosuke Kawashima, Masanori Ozaki, Masayuki Namiki, Nobutaka Inoue, Ken-ichi Hirata, Mitsuhiro Yokoyama

Abstract—Monocyte chemoattractant protein-1 (MCP-1), which binds to C-C chemokine receptor 2, has been implicated as the primary source of monocyte chemoattractant function in the early stages of atherosclerosis. Recently, propagermanium, a drug used clinically for the treatment of chronic hepatitis in Japan, has been shown to inhibit C-C chemokine receptor 2 function and suppress monocyte/macrophage infiltration in vitro and in vivo. Given the importance of monocyte infiltration in atherogenesis, the inhibition of it by propagermanium might prevent atherosclerosis. Apolipoprotein E knockout (apoE-KO) mice were fed an atherogenic high cholesterol diet with or without 0.005% propagermanium for 8 or 12 weeks. Although the plasma lipid levels were unchanged by the drug treatment, atherosclerotic lesion area in the aortic root was reduced by 50% in the drug-treated apoE-KO mice compared with the nontreated apoE-KO mice after 8 weeks of cholesterol feeding ($0.62 \pm 0.12$ versus $1.27 \pm 0.07$ mm$^2$, respectively; $P<0.01$). Moreover, the accumulation of macrophages in the lesions was markedly reduced in the drug-treated group (macrophage positive area, $0.23 \pm 0.06$ mm$^2$ [drug-treated group] versus $0.67 \pm 0.07$ mm$^2$ [control group]; $P<0.01$). After 12 weeks of cholesterol feeding, atherosclerotic lesion formation in the aortic root and in the descending thoracic aorta was significantly reduced in the drug-treated group. Inhibition of macrophage infiltration by propagermanium prevented the formation of atherosclerotic lesions in apoE-KO mice. This drug may serve as a therapeutic tool for the treatment of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2002;22:lll–lll.)

Key Words: macrophage ■ drug ■ monocyte chemoattractant protein-1 ■ C-C chemokine receptor 2 ■ atherosclerosis

Atherosclerosis is a complex chronic inflammatory disease and involves focal accumulation of lipids and inflammatory cells, smooth muscle cell proliferation and migration, and the synthesis of extracellular matrix. The infiltration of macrophages into the vascular wall and their transformation into foam cells is one of the characteristics of early atherogenesis. A number of adhesion molecules, growth factors, cytokines, and chemoattractants have been implicated in this process. One of the chemotactic factors implicated in the early atherogenesis is monocyte chemoattractant protein-1 (MCP-1), which binds to C-C chemokine receptor 2 (CCR2). MCP-1 is highly expressed in macrophage-rich regions of human and rabbit atherosclerotic lesions. CCR2 is displayed on peripheral blood monocytes, macrophages, and lymphocytes. Moreover, CCR2 expression is increased in monocytes isolated from hypercholesterolemic patients compared with those from normocholesterolemic individuals. Taken together, these previous investigations strongly suggest that MCP-1 and its receptor, CCR2, might play important roles in recruiting and retaining macrophages at atherosclerotic lesions.

An organic germanium compound, propagermanium, has been clinically used for the treatment of hepatitis B virus–induced chronic hepatitis in Japan. Recent studies have demonstrated that this drug potently inhibits the infiltration of macrophages in vivo. One of mechanisms of this drug is the inhibition of CCR2 function. Given the importance of the MCP-1/CCR2 pathway in the process of atherosclerosis, suppression of CCR2 function by propagermanium might prevent the formation of atherosclerotic lesions. To test this hypothesis, we examined the effect of propagermanium on atherosclerosis in cholesterol-fed apoE knockout (apoE-KO) mice.

Methods

Materials

Propagermanium (3-oxygemylpropinic acid polymer) was purchased from Sanwa Chemical Co. All other drugs and culture media used in the present study were purchased from Sigma Chemical Co. Normal chow and an atherogenic high cholesterol diet (consisting of a normal chow diet plus 1.25% cholesterol, 7.5% cocoa butter, 7.5% casein, and 0.5% sodium cholate) was obtained from Oriental Yeast.
Monocyte–Endothelial Cell Adhesion Assay
In Vitro
Mouse aortic endothelial cells (MAECs) were isolated, and primary culture was accomplished as previously described.1 Confluent MAECs from apoE-KO mice between the fourth and sixth passages maintained in DMEM containing 20% FBS, endothelial cell growth supplements (Becton Dickinson), and penicillin-streptomycin (GIBCO-BRL) were prepared and used for this experiment. A mouse monocyte cell line (J774.1, Cell No. RCB0434, Riken) was cultured in RPMI 1640 with 10% FBS. The adhesion of J774.1 cells to MAECs was studied according to a method modified from that described by Pawlowski et al.1 The media of J774.1 cells was changed to RPMI 1640 containing 0.5% FBS with or without 3 μg/mL propagermanium at 24 hours before the adhesion assay, and 10 nmol/L recombinant mouse JE/MCP-1 (Genzyme Techne) was added 4 hours before the experiment. MAECs were cultured until confluent in gelatin-coated 24-well tissue culture plates. The media of MAECs were changed to DMEM containing 20% hyperlipidemic serum-free DMEM 3 times, J774.1 cells (3.0 × 10⁵ per well) were added to the wells, incubated for 15 minutes at 37°C, and humidified with 5% CO2. Nonadherent cells were rinsed off with 3 washes with the use of DMEM, and the cells were fixed in 10% formaldehyde neutral buffer solution. The number of adherent cells was determined by counting a minimum of 3 fields per well by using inverted phase-contrast microscopy (Nikon) at ×200 magnification.

Assay of Infiltrated Macrophages In Vivo
To estimate the infiltration of macrophages in vivo, the thioglycollate-induced cell infiltration model was used. One millilitre thioglycollate (4% thioglycollate medium) injection in the abdominal cavity of mice induced remarkable inflammatory cell infiltration to the abdominal cavity. In this model, the MCP-1/CCR2 pathway is reported to play a crucial role in the infiltration of macrophages.14 One week before the thioglycollate injection, 28 male C57BL/6 mice were divided into 2 groups and fed normal chow without additional supplementation (control group, n = 13) or chow supplemented with 0.005% propagermanium (drug group, n = 15). At 1 day or 4 days after thioglycollate injection, abdominal cavities were washed with physiological saline, and the number of infiltrated cells was counted with a hemocytometer. Cells were applied to microscope slides with the use of a cytospin centrifuge and stained with Diff-Quik, and differential counts were obtained by morphological analysis.15 Differential cell counts were determined by counting 300 cells per slide.

Animal Preparation and Experimental Design
Forty-eight apoE-KO mice (26 male and 22 female, offspring of homozygous apoE-KO mouse mating, backcrossed onto the C57BL/6 background) were used in the present study. Those mice were weaned at 4 weeks, divided into 2 groups (control and drug-treated), and switched to the atherogenic high cholesterol diet. The mice were fed the atherogenic diet without additional supplementation (control group, n = 23) or diet supplemented with 0.005% propagermanium (drug group, n = 25). This concentration of propagermanium in the feeding chow results in a dose of 5 mg/kg per day in each mouse. This dose of the drug had no unusual effect on normal mice and never caused death in this study.10 Animals were provided with the diet and water ad libitum and maintained on a 12-hour light/dark cycle. All animal experiments were conducted according to the Guidelines for Animal Experiments at Kobe University School of Medicine.

Plasma Lipid Analysis and Plasma MCP-1 Assay
Immediately after euthanasia, blood was collected from mice by cardiac puncture. Plasma was obtained by centrifugation of the blood and stored at −80°C until each assay. Total cholesterol and triglyceride levels were determined by using an automated clinical chemical analyzer. HDL cholesterol levels were quantified by enzymatic reaction with the use of a commercially available kit (Wako). Plasma MCP-1 levels were assessed by a commercial ELISA kit (Biosource International).

Histological Analysis of Atherosclerotic Lesions
Several mice of each group were euthanized at the age of 12 or 16 weeks (8 or 12 weeks on the atherogenic diet, respectively). The proximal aortas and sections of the heart containing the aortic root were removed, equilibrated in 20% sucrose, and embedded in OCT compounds (Tissue-Tek). Cryosections (10 μm thickness) spanning 550 μm of the aortic root were cut and collected. Five sections (every 10th section, each 100 μm apart) from each mouse were stained with Sudan III and hematoxylin.1 For quantitative analysis of the atherosclerotic lesion, the total lesion area of 5 sections from each mouse was measured with the use of NIH Imaging Software according to the modified method described by Paigen et al.16

Several descending thoracic aortas from mice fed the atherogenic diet for 12 weeks (at the age of 16 weeks) were opened longitudinally along the ventral midline and fixed with 10% formaldehyde. A gross view of the thoracic aorta was observed by using a stereoscope (Olympus SZX12). The remainder of the descending thoracic aorta was removed, embedded in OCT compound, sectioned every 50 μm, and stained with Sudan III. Approximately 25 sections from each mouse were used for quantitative analysis. The percentage of atherosclerotic lesion coverage (arc length of atherosclerotic lesion was divided by total circumference length of aortic lumen) in the descending thoracic aorta was quantified by digital morphometry. In each mouse, the mean percentage of the atherosclerotic lesion coverage from each section was used for statistical analysis.

Immunohistochemistry
Serial cryosections of the aortic root collected at 100-μm intervals were stained with an anti-mouse monocyte/macrophage monoclonal rat antibody (MOMA-2, Biosource International; 1:250 dilution) or an anti-human T-cell polyclonal rabbit antibody (CD3, DAKO; 1:100 dilution), followed by detection with biotinylated secondary antibodies and streptavidin-horseradish peroxidase.6 Quantitative analysis of the positive staining area was performed as described previously, and the percentage of the positive staining area (positive staining area/total atherosclerotic lesion area) was calculated.4

Statistical Analysis
Data are presented as mean±SEM. An unpaired Student t test was used to detect significant differences when 2 groups were compared. For analysis of lesion size, comparisons between groups were performed by use of the Mann-Whitney U test. A value of P<0.05 was considered to be statistically significant.

Results

Propagermanium Inhibited MCP-1–Induced J774.1 Cell Adhesion to Mouse Endothelial Cells
In Vitro
At first, in accordance with an assay described in a previous study,13 we established a mouse monocyte–endothelial cell adhesion assay in vitro to determine the effects of propagermanium on the interaction between those cells. Propagermanium treatment did not affect the adhesion of J774.1 cells to endothelial cells (9.1±1.6 per observed field for drug group and 8.6±0.8 per observed field for control group [n = 14 and 16, respectively]; P = NS). However, the drug treatment significantly reduced the MCP-1–stimulated adhesion of these cells (27.5±1.6 per observed field for drug group and 62.9±3.3 per observed field for control group [n = 18 and 22, respectively]; P<0.01).

Propagermanium Reduced Thioglycollate-Induced Macrophage Infiltration to the Abdominal Cavity
We investigated whether propagermanium could inhibit the infiltration of macrophages in vivo by using the thioglycollate-
Effect of Propagermanium on Thioglycollate-Induced Inflammatory Cell Infiltration to the Abdominal Cavity

<table>
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<th>Total cells, ×10000 cells</th>
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<th>Macrophages, ×10000 cells</th>
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<td>1517 ± 94</td>
<td>128 ± 11</td>
<td>161 ± 16</td>
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<td>1417 ± 122</td>
<td>137 ± 15</td>
<td>155 ± 41</td>
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<td>Four days</td>
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<tr>
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<td>437 ± 76</td>
<td>2251 ± 242</td>
<td>22 ± 9</td>
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<tr>
<td>Drug</td>
<td>1466 ± 92 (9)*</td>
<td>347 ± 33</td>
<td>1082 ± 63*</td>
<td>37 ± 9</td>
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Nontreated mice (Control) and drug-treated mice (Drug) were examined at 1 and 4 days after thioglycollate injection. Total numbers of infiltrated inflammatory cells into the mouse abdominal cavity were calculated. Differential cell counts were obtained by morphological analysis and determined by counting 300 cells per slide. The number of independent experiments is shown as (n). Results are shown as mean ± SEM. *P < 0.01 versus nontreated mice (Control) at the same condition.

induced inflammatory cell infiltration model in mice. Oral administration of propagermanium significantly reduced the thioglycollate-induced macrophage infiltration to the abdominal cavity, whereas it did not affect the granulocyte and lymphocyte infiltration (Table). These data showed that this drug selectively inhibits the infiltration of macrophages in vivo.

Plasma Lipid Analysis and General Appearance of Mice

Treatment with propagermanium did not influence plasma lipid levels (for respective control versus drug group, total cholesterol 59.0 ± 2.4 versus 53.6 ± 1.7 mmol/L, triglycerides 0.09 ± 0.02 versus 0.10 ± 0.02 mmol/L, and HDL cholesterol 1.55 ± 0.16 versus 1.76 ± 0.13 mmol/L; n = 10 [control group] and 12 [drug group]; P = NS). This drug neither changed the plasma GOT and LDH levels nor affected peripheral white blood cell number and the ratio of monocytes (data not shown). This drug did not affect the plasma MCP-1 levels in apoE-KO mice fed the atherogenic diet for 8 weeks (383.6 ± 71.6 pg/mL in nontreated control group versus 389.4 ± 105.6 pg/mL in drug group; n = 5 and 7, respectively). There were no differences in the mean body weights of the 2 groups of apoE-KO mice at the age of 16 weeks (for respective control group versus drug group, 22.8 ± 0.8 versus 21.8 ± 1.4 g [male] and 17.5 ± 1.0 versus 18.4 ± 0.8 g [female]).

Reduced Atherosclerotic Lesions of ApoE-KO Mice Treated With Propagermanium

Representative hematoxylin-eosin–stained (mice aged 12 weeks) and Sudan III–stained (mice aged 16 weeks) aortic root sections from apoE-KO mice fed the atherogenic diet are shown in Figure 1. There was no difference in atherosclerotic lesion area irrespective of sex (data not shown). Generally, the lesions were less extensive in the drug group than in the control group. After 8 weeks on the atherogenic diet (mice 8 weeks)

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Light photomicrographs of aortic root in apoE-KO mice maintained on the atherogenic diet for 8 weeks (A and B) or 12 weeks (C and D). A and C, Nontreated (control) group. B and D, Propagermanium-treated (drug) group. Sections were taken at the same level of aortic valves and stained for lipid with hematoxylin and eosin (A and B) or with Sudan III (C and D). Representative images are shown. Original magnification ×80; bar = 200 μm. E, Quantitative analysis of atherosclerotic lesion in the control group (solid bar) and drug group (open bar). Total lesion area of 5 sections in the aortic root from each mouse was calculated. Values are mean ± SEM of at least 8 mice in each group. *P < 0.05 and **P < 0.01 vs control group.
aged 12 weeks), the mean lesion area was 1.27 ± 0.07 mm² in the control group. In contrast, the atherosclerotic lesion was significantly reduced to 0.62 ± 0.12 mm² (P < 0.01) in the drug group (Figure 1E). The same observation applied to the apoE-KO mice fed the atherogenic diet for 12 weeks (2.14 ± 0.09 mm² in the control group versus 1.36 ± 0.07 mm² in the drug group; P < 0.05). Lesions in the control apoE-KO mice progressed steadily with time, whereas the lesion progression in the drug group apoE-KO mice was markedly attenuated. The mean lesion size was ~50% smaller at 8 weeks and 36% smaller at 12 weeks in the drug group on the atherogenic diet than in the control apoE-KO group (Figure 1E).

Segmental atherosclerotic lesions in descending thoracic aortas were also detected in apoE-KO mice fed the atherogenic diet for 12 weeks (Figure 2). The percentage of atherosclerotic lesion coverage in descending thoracic aortas was also reduced in the drug group (6.2 ± 1.1% [drug group] versus 9.8 ± 0.9% [control group], P < 0.01; Figure 2E).

**Propagermanium Reduced Macrophage Infiltration in Atherosclerotic Lesion**

Macrophages were abundant in the subendothelial area of atherosclerotic lesions in control apoE-KO mice fed the atherogenic diet for 8 weeks (Figure 3A and 3C). In contrast, fewer macrophages were present in the lesion of the drug-treated apoE-KO mice (Figure 3B and 3D). Quantitative analysis revealed that the area of MOMA-2–positive staining was significantly reduced in the drug-treated apoE-KO mice (0.23 ± 0.06 [drug group] versus 0.67 ± 0.07 [control group] mm² per total of 5 slices, P < 0.01). Moreover, the percentage of the MOMA-2–positive area was significantly decreased in the drug-treated group (36.1 ± 5.8% [drug group] versus 52.9 ± 6.2% [control group], P < 0.01; Figure 4A), but the percentage of CD3-positive area did not change (13.6 ± 1.6% [drug group] versus 11.6 ± 1.6% [control group], P = NS; Figure 4B).

**Discussion**

Macrophage infiltration to the atherosclerotic lesion plays a central role in the initiation and progression of atherosclerosis. However, no pharmacological interventions to inhibit the infiltration of monocytes and macrophages are clinically available. In the present study, we showed that the propagermanium, which inhibits macrophage infiltration through the suppression of CCR2 function, reduces atherosclerosis in apoE-KO mice. This drug has been used as a therapeutic agent against hepatitis B virus–induced chronic hepatitis in Japan. Therefore, this drug therapy could be a new therapeutic approach to prevent atherosclerosis in humans.

Recently, it has been reported that propagermanium reduces monocyte/macrophage infiltration into the liver and inhibits liver damage in 2 different experimental mouse liver injury models. Furthermore, Yokochi et al demonstrated that this drug suppressed monocyte migration in vitro and macrophage infiltration in vivo. The inhibition of CCR2 function is shown as a working mechanism of this drug. It does not affect the function of other chemokines, such as interleukin-8 and RANTES, except for MCP-1 and MCP-3, which are ligands for CCR2. However, this drug inhibits...
neither the activity of MCP-1 nor the binding of MCP-1 to its receptor CCR2. Therefore, the drug does not act as an antagonist of MCP-1 and CCR2. We confirmed that propagermanium did not affect the plasma MCP-1 levels in cholesterol-fed apoE-KO mice. Moreover, we also verified that the drug does not affect CCR2 expression in atherosclerotic lesions of macrophages of apoE-KO mice (data not shown). Although the detailed working mechanisms of this drug are not been fully understood, it binds to the N-terminal peptides of CCR2 together with a glycosylphosphatidylinositol (GPI)-

Figure 3. Photomicrographs of immunostaining for MOMA-2 (A through D) and CD3 (E and F) of sections of aortic lesions in apoE-KO mice maintained on the atherogenic diet for 8 weeks (at the age of 12 weeks). A, C, and E, Nontreated (control) group. B, D, and F, Propagermanium-treated (drug) group. Original magnification ×80; bar=200 μm (A and B). Original magnification ×400; bar=100 μm (C through F).

Figure 4. The percentage of positive area of MOMA-2 (A) and CD3 (B) staining in nontreated (control) group (solid bar) and drug-treated (drug) group (open bar). To determine the relative amounts of macrophages and T lymphocytes in an atherosclerotic lesion, the positive area of MOMA-2 and CD3 was divided by the total lesion area of atherosclerosis. Values are mean±SEM of at least 8 mice in each group. *P<0.01 vs control group.
anchored protein and inhibits a part of CCR2 function (Yokochi et al). In the study of Yokochi et al, phosphatidylinositol–phospholipase C, which selectively cleaves the GPI-anchored protein was shown to eliminate the effects of propagermanium on CCR2 function, and this showed that the drug might act through the GPI-anchored protein. In the present study, we exhibited that this drug markedly attenuates the MCP-1–induced adhesion of J774.1 cells to the endothelium in vitro and also reduces the thioglycollate-induced macrophage infiltration to the abdominal cavity in vivo. A previous report has shown that the MCP-1/CCR2 signal pathway plays a critical role in the thioglycollate-induced macrophage infiltration model in mice. Moreover, we have shown that this drug reduces the infiltration of macrophages in atherosclerotic lesions but not the infiltration of T lymphocytes. We cannot deny the possibility that this drug affects the function of T lymphocytes and the production of other cytokines. However, the main target of propagermanium seems to be the CCR2 of monocytes and macrophages, although further investigation regarding the working mechanisms of this drug are needed. Recently, CCR2 is shown to be expressed in endothelial cells and to be related to their activation. The effect of this drug on endothelial function will be further examined.

Our data are consistent with previous reports showing that CCR2 deficiency and MCP-1 deficiency reduce atherosclerotic lesion formation in gene-engineered atherosclerosis-prone mice. A more recent report has indicated that anti–MCP-1 gene therapy using a deletion mutant of human MCP-1 gene inhibits the formation of atherosclerosis in apoE-KO mice. Taken together, the MCP-1/CCR2 pathway would be the most promising therapeutic target in the prevention of atherosclerosis. Moreover, several chemokines and cytokines have been suggested as therapeutic targets in the inhibition of atherosclerosis by modification of macrophage infiltration. It has been reported that lack of macrophage colony–stimulating factor (M-CSF) reduces atherosclerotic lesions in apoE-KO mice. Murayama et al have reported that the anti–c-fms (the receptor of M-CSF) antibody prevents early atherogenesis in apoE-KO mice. Alternatively, another chemokine, interleukin-8, and its receptor, CXC receptor-2, might be therapeutic targets against atherogenesis. However, the therapeutic interventions on these molecules may induce side effects. For example, mice lacking M-CSF have an osteoclast deficiency that results in impaired bone remodeling and skeletal deformities, and CXC receptor-2 mutant mice develop lymphadenopathies and splenomegaly. On the other hand, CCR2 mutant mice do not exhibit extreme abnormal findings under normal conditions; i.e., these mice are without abnormalities in leukocyte adhesion and migration under the inflammatory conditions. Moreover, the dose of propagermanium used in the present study is not relevant to the dose clinically used for treatment (a dose of 30 mg/d is clinically used). Thus, an intervention on the MCP-1/CCR2 pathway by this drug may be a safe and an effective therapeutic tool for atherosclerosis.

In conclusion, we have demonstrated that propagermanium attenuates atherogenesis via the inhibition of macrophage infiltration in apoE-KO mice. Although further experiments defining the effects of the drug on atherogenesis are needed, this finding could provide us with a new, unique, cell-specific therapeutic approach in the use of drugs to prevent atherosclerosis.

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References


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