Effects of MCI-186 (Edaravone), a Novel Free Radical Scavenger, Upon Experimental Atherosclerosis in Apolipoprotein E-Deficient Mice

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Background Recent evidence suggests that oxidative stress may play a role in the development of atherosclerosis. MCI-186 (3-methyl-1-phenyl-1-parazolin-5-one, edaravone) is a novel free radical scavenger, but it remains unclear whether free radical scavengers would be effective for the prevention of the disease.

Methods and Results Experimental atherosclerosis was induced in apolipoprotein E-deficient mice fed a high-fat diet containing 0.3% cholesterol. Mice were treated with an intraperitoneal injection of either MCI-186 1 mg/kg per day or MCI-186 10 mg/kg per day on alternate days over 4 weeks. Fatty streak lesion was suppressed by MCI-186 10 mg/kg per day administration, but not by mg/kg per day. Immunohistochemical analysis showed that macrophage and CD4⁺ T-cell accumulation and oxidative stress overload in the fatty streak lesion were suppressed in mice that received MCI-186 treatment.

Conclusions MCI-186 administration suppressed the development of atherosclerosis, associated with reduced expression of both immune-activated cells and oxidative stress in fatty streak plaques. (*Circ J* 2006; 70: 1216–1219)

Key Words: Atherosclerosis; Free radical scavenger; Oxidative stress

Atherosclerosis is associated with immune activation and inflammation.^{1–3} Some investigations point to inflammatory/immune activation of plaques as a cause of acute coronary syndromes^{4,5} and have also suggested links between atherosclerosis and microbial infections.^{4,6,7} Recently, it was suggested that oxidative stress may play a significant role in the development of atherosclerosis.^{8–10}

MCI-186 (3-methyl-1-phenyl-2-phyrazolin-5-one, edaravone) is a novel free radical scavenger¹¹ that may be an effective agent for reducing tissue damage by inhibiting free radical production.¹² Recently, we found that in apolipoprotein E (apo E)-deficient mice given immunoglobulin treatment (ie, immunosuppressive therapy), administered simultaneously with development of the disease, atherosclerosis was suppressed via the Fc portion.^{13,14} However, because it remains unclear whether the treatment with free radical scavengers would prevent atherosclerosis, we examined the effects of MCI-186 administration in an experimental atherosclerosis model.

Methods

Experimental Atherosclerosis

The apo E-deficient 129ola×C57BL/6 hybrid mice were the generous gift of Dr Edward M. Rubin (University of

California, Berkeley, CA, USA). They mice were mated with C57BL/6 mice to produce F₁ hybrids. The F₁ apo E^{+/−} mice were then backcrossed to C57BL/6 mice for 10 generations. Mice homogeneous for the apo E-null allele on a C57BL/6 background were subsequently generated and kept in a temperature-controlled facility on a 14.10-h light–dark cycle with free access to food and water. After being weaned at 4 weeks of age, the mice were fed a normal chow diet until 6 weeks of age, when they were switched to a high-fat diet containing 20% fat and 0.3% cholesterol, as previously described.^{13–15}

All animal experiments were performed in accordance with the Declaration of Helsinki, and were approved by the institutional ethics committee for animal experiments.

MCI-186 Treatment

At 6 weeks of age, the mice were divided into 3 groups and treated with (1) vehicle (saline, n=5), (2) low-dose MCI-186 (1 mg/kg per day, n=5) (MCI-1), or (3) high-dose MCI-186 (10 mg/kg per day, n=5) (MCI-10), intraperitoneally 3 times per week (on alternate days) for 4 weeks. The administration route and dosages were determined from previous reports.^{11,12} MCI-186 was kindly supplied by

Table 1 Physiological Parameters

	n	BW	TC (mg/dl)	TG (mg/dl)
Control	5	25.4±3.8	689.3±86.7	42.4±10.8
MCI 1 mg/kg per day	5	23.7±2.6	714.3±57.2	51.1±18.7
MCI 10 mg/kg per day	5	22.1±2.7	704.3±74.8	50.1±9.2

Data are mean±SD.

BW, body weight; TC, total cholesterol; TG, triglycerides.

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Fig 1. Effect of MCI-186 on atherosclerotic lesions. The lesions in the MCI-186-treated mice (arrows) were smaller and covered less of the inner circumference of the aortic root than those of the control mice (arrows) ($\times 40$).

Mitsubishi Wellpharma Co Ltd (Tokyo, Japan).

Tissue Processing

Mice were killed by bleeding from a puncture of the right ventricle. The blood was collected and allowed to clot. After the serum was separated, lipid profiles were analyzed. The vasculature was perfused with sterile phosphate-buffered saline. The root of the aorta was dissected under a macroscope and frozen in OCT embedding medium for serial cryosectioning covering 1.0 mm of the root. The first section was harvested when the first cusp became visible in the lumen of the aorta. Four sections of 10- μ m thickness were harvested per slide, and thus 20 slides per mouse were prepared. All sections were immersed for 2 min in 60% isopropanol, stained for 15 min in a saturated oil red-O solution at 37°C, counterstained with hematoxylin, and then mounted under coverslips with glycerol gelatin.^{13–15}

Quantitation of Atherosclerotic Lesions

The oil red-O-stained sections were analyzed at a magnification of $\times 10$, as previously described.^{13–15} The image was captured directly from the RGB camera attached to the light microscope and displayed on a microcomputer to quantify the cross-sectional surface area of the lesion and the cross-sectional surface area of the vessel. The fractional area of the lesion was calculated by dividing the whole vessel area, including the lumen, intima, media and adventitia, as previously described. For each animal, 20 sections (ie, every 4th section) were examined, and the mean of the fraction area was calculated.^{13–16}

Immunohistochemistry

Aortic root cryosections were processed for immunohistochemistry as described previously.^{13–15} In brief, anti-macrophage (anti-M ϕ , M3/84, 1:400, PharMingen), anti-CD4 (GK1.5, 1:50, PharMingen), anti-CD8 (53–6.7, 1:50, PharMingen), and anti-I-A^b (25–9-17, 1:25, PharMingen) antibodies were applied to acetone-fixed cryosections. After washing, the sections were then exposed to a second antibody (horseradish peroxidase-conjugated antibodies), and the antibody binding was visualized with diaminobenzidine. Sections were counterstained with 1% methyl green. The percentage of positively stained cells per infiltrating cells in the lesions was calculated for each antibody

Table 2 Quantitation of Atherosclerotic Lesions

	n	Lesion area, μm^2 (%)
Control	5	$84.5 \pm 16.4 \times 10^3$ (10.8 ± 2.1)
MCI 1 mg/kg per day	5	$50.1 \pm 11.0 \times 10^3$ (6.4 ± 1.4)
MCI 10 mg/kg per day	5	$39.1 \pm 13.3 \times 10^3$ (5.0 ± 1.7)*

* $p < 0.01$ vs Control. Data are mean $\pm$ SD.

Atherosclerotic lesions were suppressed in mice that received MCI-186 10 mg/kg per day.

In contrast, atherosclerotic lesions were not ameliorated in mice that received 1 mg/kg per day.

as previously described;^{13–15} that is, lesions of the aortic root were analyzed. Data were obtained by dividing the number of positively stained cells by all methyl green-stained cells inside the internal elastic lamina. Three to 5 random microscopic fields were analyzed at $\times 200$. To analyze the oxidative stress overload in the aortic wall, immunohistochemistry for thioredoxin (TRX), which is a redox-active protein and considered a marker of oxidative stress, was performed as previously described!^{7–19}

Serum Lipid Measurement

Serum was separated by centrifugation and stored at -80°C . Serum total cholesterol and triglyceride levels were measured with assay kits (Wako) according to the manufacturer's instructions.

Statistical Analysis

Values are expressed as mean $\pm$ SD. Statistical analysis of the data was determined by one-way ANOVA, followed by the Fisher protected least-significant-difference test. A value of $p < 0.05$ was considered statistically significant.

Results

Effects of MCI-186 on Physiological Parameters

As shown in Table 1, treatment of MCI-186 did not significantly modify the serum lipid profiles. In addition, the drug did not reduce or increase body weight.

Effects of MCI-186 on Fatty Streak Formation

Controls developed extensive lesions in the root of the

Table 3 Effects of MCI-186 Upon Inflammatory Cell and TRX Expression in Atherosclerotic Lesions

	Control	MCI-186 1 mg/kg per day	MCI-186 10 mg/kg per day
<i>M</i> φ (%)	23.3±4.9	20.8±8.2	15.0±7.2*
CD4 ⁺ (%)	28.0±10.2	22.3±7.6	16.7±5.9*
CD8 ⁺ (%)	9.2±3.6	7.7±3.3	5.9±4.7
I-A ^{b+} (%)	4.8±2.1	3.9±2.5	4.4±3.0
TRX	++++	++	+

**p*<0.05 vs Control. Data are mean±SD.

*M*φ, macrophage; TRX, thioredoxin.

Data were obtained by dividing the number of positively stained cells by all methyl green-stained cells inside the internal elastic lamina. Three to 5 random microscopic fields were analyzed at ×200. Treatment with MCI-186 10 mg/kg per day, but not 1 mg/kg per day, reduced the expression of both macrophages and CD4⁺ cells in the lesions.

TRX expression was suppressed by MCI-186 treatment. The grading of TRX expression was done semiquantitatively. Representative data from 3 distinct trials are shown.

Fig 2. Effects of MCI-186 on oxidative stress. The expression of thioredoxin (TRX) in the lesions of the MCI-186-treated mice was decreased compared with control mice. Brown staining (arrowheads) shows the positive area for TRX expression in the atherosclerotic plaques (×200).

aorta. Fatty streak lesions were suppressed by MCI-186 10 mg/kg per day administration, but not by 1 mg/kg per day (Fig 1). In mice treated with MCI-186 10 mg/kg per day, the fractional area of the lesion was significantly reduced (Table 2).

Effects of MCI-186 on Expression of Inflammatory Cells and Oxidative Stress in the Aortic Wall

Macrophage and CD4⁺ cell accumulation in the fatty streak lesions was suppressed in mice receiving MCI-186 10 mg/kg per day but not in those receiving 1 mg/kg per day (Table 3). TRX expression in the aortic walls was suppressed by MCI-186 treatment (Table 3, Fig 2).

Discussion

The present findings clearly demonstrated that MCI-186, a novel free radical scavenger, suppressed the severity of atherosclerosis in apoE-deficient mice, and that the atheroprotection conferred by MCI-186 may be related to its scavenging for free radicals in the aortic walls, possibly by the modification of oxidative stress.

Several studies have described the participation of free radicals in the pathogenesis of atherosclerosis^{8–10} In the present study, MCI-186 treatment suppressed the severity of experimental atherosclerosis. Immunohistochemical study showed that the intensity of TRX staining in the aortic wall was reduced by MCI-186 treatment. It has already

been established that the degree of tissue TRX staining correlates with oxidative stress overload!^{7–19} Accordingly, MCI-186 treatment of apoE-deficient mice protects against atherosclerosis by reducing oxidative stress.

In addition, MCI-186 suppressed not only the severity of atherosclerosis but also the inflammatory cell infiltrates in the fatty streak plaques. Recent evidence suggests that reactive oxygen species are involved in the antigen-presenting function of dendritic cells and immune effector cells, and that antioxidants suppress the activation of these cells²⁰ In another clinical model of myocarditis, it was reported that dendritic cells play a crucial role in the autoimmune process for the development of the disease.²¹ Accordingly, MCI-186 treatment resulted in a reduction of the autoimmune mechanisms by suppressing antigen-presentation by immune effector cells.

To date, there are no distinct protective agents for the prevention of atherosclerosis in the clinical setting. We have already reported that immunoglobulin administration, beginning simultaneously with the development of the disease, suppressed the lesions of an experimental atherosclerosis via the Fc portion.¹³ Recent studies suggest that one mode of action of immunoglobulins is via the Fc receptor IIB, an inhibitory receptor, and that a large dose of immunoglobulins increases the expression of the Fc receptor IIB.^{22,23} We have confirmed that MCI-186 protects against acute murine experimental myocarditis by its free radical scavenging action, especially the hydroxyl radical scaveng-

ing action²⁴ There is increasing evidence to support the critical role of free radicals in the development of atherosclerosis^{8–10} and the rationale for prescribing MCI-186 in patients with atherosclerosis is based on its radical scavenging action and capacity to reduce immune-activated cell accumulation in the lesions. In the present study, MCI-186 treatment for 4 weeks ameliorated the experimental atherosclerosis. Accordingly, although there are some difficulties in practical use between the clinical and experimental settings, exploration of the usefulness of free radical scavengers in the treatment of atherosclerosis in humans appears warranted.

In conclusion, free radicals may be involved in the development of atherosclerosis. MCI-186 protected against experimental atherosclerosis in apoE-deficient mice by scavenging free radicals, resulting in reduced oxidative stress.

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