Inhibition of Neointimal Hyperplasia Development by MCI-186 is Correlated With Downregulation of Nuclear Factor-κB Pathway

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Background  Atherosclerosis is a progressing inflammatory process that leads to the activation of endothelial cells (ECs), monocytes and smooth muscle cells (SMC)1–4. The initial step, resulting in inflammatory response, is the sensitization of ECs by the oxidative modification of low-density lipoprotein, followed by the production of chemokines, pro-inflammatory cytokines and the expression of adhesion molecules, which is concomitant with the migration of monocytes into subendothelial spaces3–5. These monocytes differentiate into macrophages that then accumulate by scavenging lipids and, eventually, the lipid-laden macrophages have to undergo cell turnover through apoptosis.6,7 Furthermore, the migration and proliferation of SMC proceeds to neointimal hyperplasia and fibrous plaque formation8. In addition, it has been reported that oxidative stress accelerates the progression of atherosclerosis9. Free radicals, including reactive oxygen species (ROS) and reactive nitrogen species, are produced by the activation of nicotinamide adenine dinucleotide phosphate oxidase, the expression of iNOS and the secondary influence of IL-1adenine dinucleotide phosphate oxidase, the expression of gen species, are produced by the activation of nicotinamide including reactive oxygen species (ROS) and reactive nitro-

Methods and Results  Balloon injury of intima was performed in the right common carotid artery of Japanese male white rabbits, which were then fed a 1% high cholesterol diet for 4 weeks, after assigning them to either the control (n=7) or MCI-186 (0.5 mg·kg–1·day–1, n=7) group. Histological analysis revealed a reduction in neointimal thickness and lipid deposition in the subendothelial area of the MCI-186 group. Immunohistochemical analysis revealed attenuation of E-selectin expression, macrophage migration and proliferation of smooth muscle cells in the MCI-186 treated group. In in vitro studies, rabbit aorta smooth muscle cells were incubated with rIL-1 in either the presence or absence of MCI-186. MCI-186 significantly inhibited rIL-1β-induced proliferation of smooth muscle cells from rabbit aorta, as well as the activation of NF-κB. Moreover, western blot analysis showed the inhibitory action of MCI-186 on the nuclear translocation of NF-κB in human umbilical vein endothelial cells under rIL-1β stimulation.

Conclusions  MCI-186 could provide a novel therapeutic strategy for atherosclerosis by inhibiting the NF-κB pathway. (Circ J 2008; 72: 800–806)

Key Words: Antioxidants; Atherosclerosis; Free radicals

Atherosclerosis is a progressing inflammatory process that leads to the activation of endothelial cells (ECs), monocytes and smooth muscle cells (SMC)1–4. The initial step, resulting in inflammatory response, is the sensitization of ECs by the oxidative modification of low-density lipoprotein, followed by the production of chemokines, pro-inflammatory cytokines and the expression of adhesion molecules, which is concomitant with the migration of monocytes into subendothelial spaces3–5. These monocytes differentiate into macrophages that then accumulate by scavenging lipids and, eventually, the lipid-laden macrophages have to undergo cell turnover through apoptosis6,7. Furthermore, the migration and proliferation of SMC proceeds to neointimal hyperplasia and fibrous plaque formation8. In addition, it has been reported that oxidative stress accelerates the progression of atherosclerosis9. Free radicals, including reactive oxygen species (ROS) and reactive nitrogen species, are produced by the activation of nicotinamide adenine dinucleotide phosphate oxidase, the expression of iNOS and the secondary influence of IL-1β and TNF-α in ECs, SMC and macrophages mediating various signaling pathways10. Nuclear factor-κB (NF-κB) is a redox-sensitive transcription factor involved in the inflammatory response to oxidative stress by regulating inflammatory genes and apoptosis11. The rel/NF-κB family comprises 5 subunits; that is, relA (p65), rel B, c-rel, p50 and p52, which form either homo or hetero dimers and are characterized by the presence of a common N-terminal ‘rel’ homology domain. Especially, p65 has a high transcription potential to induce target genes and, moreover, p65/p50 is abundantly present in cytoplasm. In non-stimulated cells, NF-κB exists as an inactive form along with an inhibitor of NF-κB (IκB) in cytoplasm. Under inflammatory and oxidative stimulus, IκB, which is a well-elucidated molecule of the NF-κB family, undergoes phosphorylation by IκB kinase and ubiquitinylates, thus leaving the p65/p50 form of NF-κB in an activated state that then translocates into the nucleus and expresses various genes12. The activation of NF-κB progresses as a downstream in the signaling pathway involved in atherosclerosis, including modified lipoproteins, production of cytokines, adhesion molecules such as E-selectin, and SMC proliferation13. Therefore, NF-κB is universally accepted as an important target for the inhibition of atherosclerosis associated with oxidative stress14,15.

MCI-186 (3-methyl-1-phenyl-2-pyrazolin-5-one; Radicut®, edaravone), a potent free-radical scavenger, is currently being used in the management of acute ischemic stroke and also exhibits protective effects on myocardial infarction16,17.
Previous studies have demonstrated the effect of MCI-186 in attenuating ROS-induced endothelial damage, protecting against ischemia reperfusion injury and inhibiting tumor cell growth.\(^1\)\(^2\)\(^3\)\(^4\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\) Recently, MCI-186 has been reported to possess anti-atherosclerotic effects; however, the mechanism is still not known.\(^11\)\(^12\) In the present study, using a rabbit balloon injury model, we examined the hypothesis that MCI-186 attenuates the progression of atherosclerosis and the mechanism of anti-atherosclerotic effect could be explained by inhibiting the signaling pathways, such as NF-κB.

**Methods**

**Rabbit Balloon Injury Model**

Male Japanese white rabbits weighing 3–3.2 kg (Japan SLC, Inc, Shizuoka, Japan) were used. All animal experimental protocols were followed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the guidelines of our university. Anesthesia was achieved by the rabbits inhaling 1.39% isofluorane with ketamine hydrochloride (20 mg/kg), and pentobarbital sodium (50 mg/kg) as the pre-anesthetic medication. Balloon injury was performed in the right common carotid artery (RCCA) using a 3-Fr Fogarty catheter (Edwards Lifesciences, Irvine, CA, USA) and inflating the balloon 3 times at 3 atm.\(^1\) Animals were divided into 2 groups: control group (without treatment, n=7) and MCI-186 group (0.5 mg·kg\(^{-1}\)·day\(^{-1}\) iv, for 4 weeks, n=7; Mitsubishi Pharma Corporation, Tokyo, Japan) and then fed a 1% high cholesterol diet (Oriental Yeast Co, Chiba, Japan) for 4 weeks. Animals were then killed, and their RCCA were harvested and fixed with either 10% formaldehyde or embedded in OCT compound (Tissue-Tek, Sakura Finetechnical, Tokyo, Japan) for histopathological analysis.

**Histochemical and Immunohistochemical Analysis**

Paraffin sections (4 μm) were stained with hematoxylin and eosin to analyze the area of neointima and media, and cryostat-frozen sections (6 μm) were processed with Oil Red O stain to quantify lipid deposition. The sections were subjected to citrate buffer (0.01 mol/L sodium citrate and 1.39% isofluorane with ketamine hydrochloride (20 mg/kg), and pentobarbital sodium (50 mg/kg) as the pre-anesthetic medication. Balloon injury was performed in the right common carotid artery (RCCA) using a 3-Fr Fogarty catheter (Edwards Lifesciences, Irvine, CA, USA) and inflating the balloon 3 times at 3 atm.\(^1\) Animals were divided into 2 groups: control group (without treatment, n=7) and MCI-186 group (0.5 mg·kg\(^{-1}\)·day\(^{-1}\) iv, for 4 weeks, n=7; Mitsubishi Pharma Corporation, Tokyo, Japan) and then fed a 1% high cholesterol diet (Oriental Yeast Co, Chiba, Japan) for 4 weeks. Animals were then killed, and their RCCA were harvested and fixed with either 10% formaldehyde or embedded in OCT compound (Tissue-Tek, Sakura Finetechnical, Tokyo, Japan) for histopathological analysis.

**Immunocytochemistry**

Immunocytochemistry was performed to identify the nuclear translocation of NF-κB. The RASM were seeded at 0.5×10\(^4\) cells/well in 24-well Lab-Tek chamber slides (Nalge Nunc Int) and incubated overnight. The RASM were pre-incubated with or without 6 μmol/L MCI-186 for 30 min, followed by stimulation with IL-1β (30 ng/ml) for 1 h. Cells were fixed with 10% paraformaldehyde buffer for 10 min and incubated overnight with p65 NF-κB Ab (1:50) at 4°C, and then further incubated with fluorescein goat anti-rabbit IgG (H+L) secondary antibody (1:200) for 10 min at room temperature.

**Superoxide Measurement in Arteries**

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate the in situ production of superoxide from 30-μm frozen tissue sections, as described elsewhere.\(^2\)\(^3\) Cytoxic DHE exhibits blue fluorescence, but once it is oxidized by superoxide to ethidium bromide, it intercalates within the cell’s DNA, staining its nucleus a fluorescent red (excitation at 488 nm, emission 610 nm). Serial sections (30 μm) were equilibrated under identical conditions for 30 min at 37°C in Krebs–HEPES buffer. Fresh buffer containing DHE (2 μmol/L) was applied topically onto each tissue section, coverslipped and incubated for 30 min in a light-protected, humidified chamber at 37°C, and then viewed by fluorescent confocal microscopy (Bio-Rad MRC-1024), using the same imaging settings in each case.

**Western Blot Analysis**

Western blot was used to analyze the activation of ASK-1 and NFκB. Human umbilical vein ECs (HUVEC) were cultured in 35-cm\(^2\) flasks and maintained in EC medium (EBM-2; Cambrex Bio Science Walkersville, Inc, Walkersville, MD, USA) supplemented with 5% FBS, hydrocortisone 0.2 ml, hFGF-2 2 ml, VEGF 0.5 ml, R3 IGF-1 0.5 ml, ascorbic acid 0.5 ml, hEGF 0.5 ml and GA-1000 0.5 ml. The HUVEC (3×10\(^6\)) were transferred into 35-cm\(^2\) dishes and incubated overnight at 37°C with 5% CO\(_2\). To demonstrate the effect of MCI-186 on the NF-κB pathway, the HUVEC...
Fig 1. MCI-186 inhibited neointimal thickness in rabbit right common carotid artery (RCCA). The black arrows span the neointimal thickness and the blue arrows point to the media. (A) Increased neointimal thickness in the control group, and (B) neointimal thickness is reduced with MCI-186. Graphs show the ratio of (C) neointimal thickness/media thickness and (D) neointimal thickness/entire vascular. In the group treated with MCI-186, neointimal thickness was inhibited (p<0.05 vs control group). Staining by smooth muscle cell-specific \( \alpha \)-actin of the RCCA section from the (E) control group and (F) group treated with MCI-186.

Fig 2. Inhibition of E-selectin expression by MCI-186. MCI-186 inhibited E-selectin expression on endothelial cells (ECs). (A) Immunofluorescent staining of rabbit right common carotid artery (RCCA) with anti E-selectin antibody. MCI-186 inhibited E-selectin expression on balloon-injured arteries. (B) Double immunofluorescence staining was used to localize the co-expression of E-selectin and CD31. RCCA sections from the control group revealed overexpression of E-selectin on ECs, whereas expression of E-selectin was not observed in the group treated with MCI-186. Red fluorescence corresponds to E-selectin expression.
were stimulated by IL-1β (30 ng/ml) either with or without pre-incubation of 6 μmol/L MCI-186. Nuclear and cytoplasmic proteins were obtained using the method described elsewhere. Cell pellets were suspended in 100 μl buffer A (10 mmol/L HEPES, 10 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol and 0.5 mmol/L PMSF) for 15 min. Cells were then lysed by adding 10% Nonidet P-40 (25 μl) and vigorously vortexed for 10 s. Cytoplasmic protein was collected by centrifugation at 14,000 rpm for 2 min and stored at -80°C. Nuclear pellets were resuspended in 50 μl of buffer B (20 mmol/L HEPES, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol and 0.5 mmol/L PMSF) for 20 min. Nuclear protein was collected by centrifugation at 14,500 rpm for 5 min and stored at -80°C. Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Samples (20 μg) were fractionated by SDS-PAGE for NFκB, and Phospho-IκBα, and then transferred to PVDF membranes (Millipore Co, Bedford, MA, USA). The membranes were incubated with NFκB p65 rabbit polyclonal Ab (1:4,000), and phospho-IκBα (Ser32/36) mouse monoclonal Ab (1:2,000; Cell Signaling Technology, Inc, Danvers, MA, USA), followed by anti-rabbit IgG Ab and anti-mouse IgG Ab conjugated with horseradish peroxidase (SantaCruz Biotechnology). We detected protein bands using chemiluminescence reagent Amersham ECL plus (GE Healthcare, Buckinghamshire, UK).

**Quantitative Histopathology**

Neointimal thickness, neointimal lipid deposition and macrophage migration were determined using NIH image software. Neointimal thickness was calculated from the ratio of neointima/media and neointima/lumen. Lipid deposition and macrophage migration was calculated as the ratio of positively stained areas compared to the respective neointimal space. Three cross-sections were made for each RCCA specimen and examined. The average ratio ± SEM is presented.

**Statistical Analysis**

All results are expressed as the mean ± SEM. Group differences for continuous variables were determined by analysis using Student’s t-test. P-values of < 0.05 were considered statistically significant.
Results

MCI-186 Prevented the Progression of Atherosclerosis In Vivo in the Rabbit Balloon Injury Model

As shown in Figs 1A,B,E,F, neointimal thickness was detected in the RCCA of the control group, with the major constituent found to be SMC, which was determined by the presence of α-SMC actin-positive cells (Figs 1E,F). In the control group, the ratio of neointimal thickness/medial thickness was 0.468±0.08 and MCI-186 treatment decreased the ratio to 0.187±0.01 in the experimental group (p<0.02 vs control group; Fig 1C). Furthermore, the ratio of neointimal thickness/vascular lumen was 0.698±0.09 in the control group and 0.25±0.01 in the experimental group (p<0.01 vs control group; Fig 1D), and the lipid deposition ratio in the control group was 0.13±0.02 and decreased to 0.06±0.003 in the experimental group (p<0.05 vs control group; Fig 3C). The value of PCNA in areas with neointimal formation was directly proportional to cell growth, as demonstrated by the decreased cell growth after the addition of MCI-186 (Fig 4B).

MCI-186 Treatment Inhibited the Inflammatory Response to Balloon Injury

MCI-186 also reduced the production of superoxide anion in balloon-injured arteries, whereas SMC of the control arteries stained positive for the superoxide anion, as confirmed by DHE staining (Fig 3A). Since neointimal thickness was inhibited by MCI-186, we examined its possible role in the inhibition of macrophage migration. As shown in Fig 3B, in the control group, macrophage migration into the neointimal areas was detected (19.9±6.3%), whereas in the MCI-186-treated experimental group, the migration of macrophages was inhibited (0.26±0.14%) (p<0.05 vs control group). MCI-186 significantly attenuated the expression of E-selectin in injured arteries, as confirmed by immunofluorescent staining (Fig 2).

Serum Cholesterol Level Unchanged by MCI-186

Although MCI-186 inhibited the development of neointimal thickness, lipid deposition and macrophage migration, total cholesterol levels were not reduced. After 4 weeks, the total cholesterol level increased from 24.57±4.32 mg/dl to 1,205.8±153.5 mg/dl in the control group and from 28.67±7.1 mg/dl to 1,185.8±199 mg/dl in the experimental group. The differences between the 2 groups at the starting (p=0.29 vs control) and endpoint (p=0.47 vs control) were not significantly different. Lipid peroxidation levels remained unchanged in both groups (data not shown).

MCI-186 Inhibited IL-1β-Induced RASMC Growth In Vitro

IL-1β induced dose-dependent growth of RASMC, in concentrations ranging from 3 ng/ml (p<0.05 vs 10% FBS) to 30 ng/ml (p<0.01 vs 10% FBS), and yet cell viability remained unchanged (data not shown). In 10% FBS-supplemented medium without IL-1β, the number of cells increased 1.3±0.03 times compared with the starting point, whereas the addition of IL-1β increased RASMC growth by 2.6±0.29-fold. Without IL-1β stimulation, MCI-186 did affect cell growth, whereas with IL-1β, MCI-186 inhibited growth increase to only 1.39±0.16-fold with 0.6 µmol/L (p<0.01 vs IL-1β alone) and 1.35±0.15-fold with 6 µmol/L of MCI-186 (p<0.01 vs IL-1β alone) (Fig 4A). As the differ-

Fig 4. Attenuation of cell proliferation by MCI-186. (A) MCI-186 inhibited IL-1β-induced proliferation of smooth muscle cells from rabbit aorta (RASMC). The graph shows the effect of MCI-186 on RASMC proliferation when stimulated by IL-1β. Lane 1, cells with FBS only; Lanes 2 and 3, cells with MCI-186; Lane 4, cells with IL-1β only; Lanes 5 and 6, cells with MCI-186 and IL-1β. MCI-186 significantly attenuated IL-1β-induced RASMC proliferation. (B) Cell proliferation in a balloon-injured artery was examined by immunostaining with anti-proliferating cell nuclear antigen (PCNA) antibody. The number of PCNA-positive cells decreased in the experimental group compared with the control group. The PCNA index was calculated from 5 different locations in each section for each group. SMC, smooth muscle cells; FBS, fetal bovine serum.
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ence in inhibition between the 2 doses was not statistically significant, 6 μmol/L was used as the dose for MCI-186 in the experiments that followed, as it corresponds to the dosage used clinically in acute cerebral ischemia.

**MCI-186 Inhibited the Activation of NF-κB**

We attempted to determine whether MCI-186 has any effect on NF-κB activation in vivo as well. In the control group, we detected nuclear translocation of p65 NF-κB in the neointimal areas (Fig 5A), whereas it was not observed in the experimental group (Fig 5A). We examined whether RASMC proliferation was inhibited through an IL-1β-induced NF-κB activation pathway (Fig 5B). Without IL-1β, the rate of nuclear translocation of p65 NF-κB was 2±1.22%, which increased to 19.82±3.75% with 30 ng/ml of IL-1β. The addition of MCI-186 treatment reduced the IL-1β-induced nuclear translocation rate to 4.13±1.08% (p<0.01 vs IL-1β alone). This result was further confirmed by western blotting using HUVEC stimulated by IL-1β in the presence or absence of MCI-186 (Fig 5C). Furthermore, MCI-186 blocks NF-κB activation in the phosphorylation stage of IκB, thereby preventing the nuclear translocation of NF-κB. These results showed that MCI-186 inhibits the nuclear translocation of p65 NF-κB in vivo, as well as in vitro.

**Discussion**

The major findings of the present study revealed that MCI-186 treatment reduces the inflammatory response and progression of neointimal hyperplasia in balloon-injured rabbits fed a 1% high cholesterol diet for 1 month. In vivo experiments revealed a reduction in neointimal thickness, migration of macrophages and E-selectin expression, and attenuated superoxide production and lipid deposition in MCI-186-treated groups. In vitro experiments revealed reduced proliferation of SMC and the inhibition of NF-κB activation in the MCI-186 treated groups. NF-κB is involved in the regulation of genes responsible for SMC proliferation, migration of SMC into the injured site, expression of EC adhesion molecules and, thereby,

![Fig 5. MCI-186 inhibited the activation of nuclear factor κB (NF-κB). (A) In the control group, nuclear translocation of p65 NF-κB was detectable, whereas it was not seen in the experimental group B. (B) Representative immunocytochemical analysis revealed localization of p65 in the nucleus when stimulated by IL-1β, whereas MCI-186 prevented nuclear translocation. (C) Human umbilical vein endothelial cells were treated with 100 IU of IL-1β alone or in combination with 6 μmol/L of MCI-186. NF-κB and phosphorylated IκBα protein levels were measured after 1 h by western blot analysis. IκB kinase was used as an internal control. MCI-186 inhibited the nuclear translocation of p65 NF-κB under IL-1β stimulation by inhibiting the phosphorylation of IκBα.]
transformation of monocytes into macrophages. In the present study, we measured the effect of MCI-186 on NF-κB activation. Both our in vitro and in vivo data showed that MCI-186 inhibited NF-κB nuclear translocation. Free radicals and inflammatory cytokines are the causative agents of NF-κB activation. MCI-186 is a known antioxidant that scavenges for free radicals and, thereby, the nuclear translocation of NF-κB is inhibited.

Extensive proliferation of vascular SMC is considered to be the hallmark of atherosclerosis. The genes that lead to the expression of factors that is responsible for cell proliferation is regulated by NF-κB. In the present study, MCI-186 prevented the activation of NF-κB and, moreover, resulted in decreased neointimal thickness in balloon-injured arteries and decreased proliferation in SMC stimulated by IL-1. Expression of adhesion molecules is an important step in the chemotaxis of monocytes into the arterial intima. In the present study, we measured the expression of E-selectin in injured arteries. E-selectin expression was not observed in the MCI-186-treated rabbits. Being an inhibitor of NF-κB, MCI-186 could have attenuated E-selectin expression.

As a radical scavenger, MCI-186 absorbs free radicals, reduces superoxide production in balloon-injured arteries, reduces chronic stimulation of ECs and causes the down-regulation of expression of adhesion molecules, leading to decreased monocyte migration. Hence, the differentiation of monocytes into macrophages is inhibited. This might have resulted in reduced neointimal thickness with decreased SMC proliferation and migration.

Although statins and anti-hypertensive drugs are effective in preventing atherosclerosis, secondary measures, such as anti-inflammatory agents and antioxidants, also have proved beneficial. In this aspect, MCI-186 will be a useful drug in preventing early, as well as late, stages of atherosclerosis by attenuating the expression of adhesion molecules and SMC proliferation. Restenosis is the end result of the reactive proliferation of cells of the vessel wall after angioplasty. Thus, it is clear that an agent that suppresses cell proliferation may suppress restenosis. Since MCI-186 significantly prevents neointimal proliferation, it could be a prospective agent for preventing re-occlusion after percutaneous coronary intervention (PCI). MCI-186 can be used in adjunction with anti-thrombolic drugs, such as aspirin and ticlopidine, after PCI. In conclusion, MCI-186, as well as its antioxidative action, possesses anti-inflammatory properties, such as inhibiting the NF-κB molecule. To our knowledge, this is the first study that demonstrates the mechanism of inhibition of neointimal hyperplasia in balloon-injured rabbits by the radical scavenger MCI-186.

References