A Novel IKK Inhibitor Prevents Progression of Restenosis After Arterial Injury in Mice

Rikuta Hamaya, BS, Masahito Ogawa, MS, Naho Kobayashi, DDS, Jun-ichi Suzuki, MD, Akiko Itai, PhD, Yasunobu Hirata, MD, Ryozo Nagai, MD, and Mitsuki Isobe, MD

Summary

Restenosis after percutaneous coronary intervention (PCI) is still a clinically serious problem. We examined the treatment efficacy of IMD-0354, a novel IKK inhibitor, on arteriopathy. Using C57BL/6J mice, a wire-injury model was prepared and the mice were intraperitoneally injected with IMD-0354 or vehicle twice a day. The vehicle-treated injured arteries showed significantly thickened intima (3.77 ± 0.59, n = 8), however, IMD-0354 suppressed its progression (1.62 ± 0.22, n = 10, P < 0.05) on day 28. While enhanced expression of PCNA and NF-κB was observed in the untreated injured arteries, IMD-0354 significantly suppressed their expressions. Quantitative RT-PCR revealed that the expression of several inflammatory factors was reduced in the arteries from mice which received IMD-0354 treatment compared with the control animals. Thus, this drug may effectively prevent restenosis after coronary intervention and other cardiovascular diseases. (Int Heart J 2012; 53: 133-138)

Key words: IKK inhibitor, Chemical compound, Arterial remodeling, Inflammation, Smooth muscle

Restenosis, which consists of neointimal formation after percutaneous coronary intervention (PCI), is a clinically serious problem. Because inflammation is an essential pathological feature of neointimal formation, nuclear factor-kappa B (NF-κB) plays an important role in this process. In humans, activated NF-κB is detected in restenotic lesions. Thus, NF-κB regulation has the potential to suppress its progression. Activation of NF-κB induces gene expression that leads to transactivation of adhesion molecules, cytokines and chemokines, promoting the inflammatory status involved in arterial injury. NF-κB is a dimer of the Rel family members and the most common active form is composed of p50 or p52 and p65. In resting cells, NF-κB is inactive and segregated in the cytoplasm, and bound to an inhibitory protein known as the inhibitor of NF-κB (IκB). NF-κB activation requires phosphorylation of IκB by IκB kinase (IKK) complex. The phosphorylated IκB is then ubiquitinated and degraded by proteasomes. Subsequently, the unbound NF-κB is translocated to the nucleus and binds to the promoter or enhancer of specific genes, including those involved in inflammatory reactions.

Recently, the first clinical use of an NF-κB decoy oligonucleotide at the site of coronary stenting for the prevention of restenosis was reported. However, the indispensable role played by NF-κB in many biological processes has raised the concern that a complete shutdown of this pathway would have significant detrimental effects on normal cellular function. Instead, drugs that selectively target only inflammation-induced NF-κB activity would be of greater therapeutic value. IMD-0354, a novel NF-κB inhibitor, is a drug that specifically suppresses IKK-β activity. In this study, we investigated the effects of IMD-0354 on neointimal formation in vivo and VSMC proliferation in vitro.

Methods

Arterial injury models in mice: Male mice (C57BL/6, age 6-8 weeks, 20-25 g; Japan Clea, Co.) were used in this study. We prepared an arterial injury model that was modified from that of a previous report. Briefly, the femoral artery was looped and tied off with 6-0 silk sutures for temporary vascular control during the procedure. A transverse arteriotomy was made and a flexible angioplasty guidewire (a curved 350 μm polished copper wire) was introduced and advanced 1 cm. Endothelial denudation injury of the artery was performed by use of wire withdrawal injury and 3 passes were made along the artery. A sham operation (no wire injury) was also performed. Treatment protocols: IMD-0354 (N-(3,5-Bis-trifluoromethylphenyl)-5-chloro-2-hydroxy-benzamide) was used in this study (Institute of Medicinal Molecular Design Inc, Tokyo). The drug was dissolved in 0.5% carboxymethylcellulose

From the 1 Department of Cardiovascular Medicine, Tokyo Medical and Dental University, 2 Department of Advanced Clinical Science and Therapeutics, The University of Tokyo, 3 Department of Periodontology, Tokyo Medical and Dental University, 4 Institute of Medicinal Molecular Design, and 5 Department of Cardiovascular Medicine, The University of Tokyo, Tokyo, Japan. This study was supported by a Mitsui Life Insurance Research Grant, Daiwa Security Health Insurance Research Grant, Mitsui Sumitomo Marine Welfare Research Grant, Human Health Future Research Grant, a grant from the Research Foundation for Pharmaceutical Sciences, and the Japan Society for the Promotion of Science (JSPS) through its “Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program)”. Address for correspondence: Jun-ichi Suzuki, MD, Department of Advanced Clinical Science and Therapeutics, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Received for publication October 21, 2011. Revised and accepted December 16, 2011.
(CMC) vehicle immediately before use. Drug-free vehicle (0.5% CMC solution) was used as a control.

The animals were assigned randomly to one of three treatment groups, as follows: (a) intraperitoneal injection of IMD-0354 (10 mg/kg) every 12 hours to day 28; (b) intraperitoneal injection of IMD-0354 (20 mg/kg) every 12 hours to day 28; (c) intraperitoneal injection of CMC every 12 hours to day 28. The concentrations of the drug were determined according to previous reports.9,11

Quantitative assessment of arterial neointima: Tissue sections were stained with hematoxylin and eosin (HE) and Elastica van Gieson (EvG). Light microscopic morphometric computer analysis was carried out. The severity of intimal thickening was quantitatively assessed in each artery as described earlier.13 Vascular area within the external elastic lamina (EELA) and the internal elastic lamina (IELA) as well as the lumen area (LA) were measured. The I/M ratio was calculated as: I/M=(IELA−LA)/(EELA−IELA).

Immunohistochemistry: Complete transverse sections of the arteries approximately 3 mm in length were obtained and stored in an optimum cutting temperature compound (Ted Pella, Inc., Redding, CA). Serial sections (6 μm) were cut and dipped in cold acetone for 10 minutes. The sections were rehydrated in phosphate buffered saline (PBS) and incubated with 5% normal goat serum to avoid nonspecific reaction. The samples were incubated with primary antibodies against proliferating cell antigen (PCNA) (PC-10) for 12 hours at 4°C. The antibodies were detected with a horseradish peroxidase complex (Nichirei, Tokyo) according to the manufacturer’s instructions. Finally, each section was reacted with AEC solution for 5–20 minutes. Immunostained type- and class-matched nonimmune PBS was used as the negative controls for each antibody. Immunohistochemical analyses were performed by counting the number of positive cells.

Immunofluorescence: Cross-sections (6 μm) of injured femoral arteries were stained with antibodies against α-smooth muscle actin (SMA) (1A4), CD11b (M1/70), NF-κB p65 (C-20) followed by incubation with fluorescently labeled secondary antibodies. Images were obtained using a confocal microscope. Nuclei were stained using Hoechst 33342.

Real-time polymerase chain reaction (RT-PCR): We extracted mRNA from injured arteries as previously described.10 RT-PCR was used to determine the messenger ribonucleic acid (mRNA) expression of tumor necrosis factor-alpha (TNF-α; Mm00443258_m1), interleukin-6 (IL-6; Mm00446190_m1), cyclooxygenase-2 (COX-2; Mm00478374_m1), monocyte chemoattractant protein-1 (MCP-1; Mm00441242_m1), intercellular adhesion molecule-1 (ICAM-1; Mm00516023_m1), matrix metalloproteinase-3 (MMP-3; Mm00440295_m1) and plasminogen activator inhibitor-1 (PAI-1; Mm00435858_m1). To account for differences in cDNA preparation and cDNA amplification efficiency, the mRNA expression of the target gene was normalized by 18s ribosomal RNA (4308329). Quantitative data were calculated using the comparative CT method.14

Western blot analysis: Equal amounts of protein were loaded and separated by SDS-PAGE transferred to nitrocellulose membrane, and incubated with monoclonal antibody to PAI-1 (ab28207) and β-actin (AC-15). The membranes were incubated with secondary antibody and developed with enhanced chemiluminescence reagent (Amersham Biosciences). Enhanced chemiluminescence was detected with an LAS-1000 analyzer system (Fujifilm, Tokyo). Quantitative data were calculated by optical densities using image J software.

Cell preparations: Primary VSMCs were established after isolating the cells from the thoracic aortas of 7 week-old mice. Primary VSMCs were expanded and incubated in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum and penicillin at 37°C in a 5% CO2 incubator.

The spleens were removed immediately after the mice were sacrificed. A single cell-suspension was prepared by disrupting the organ in RPMI 1640 medium to obtain splenic macrophages.15 Macrophages were incubated in the same medium with 10% fetal bovine serum and penicillin at 37°C in a 5% CO2 incubator in all experiments.

Cell proliferation assay: The VSMCs were seeded on 96-well plates and then incubated with DMEM supplemented 0.5% FBS and PDGF-BB, TNF-α, or IL-1β for 24 hours in an incubator. The effects of IMD-0354 on VSMC proliferation were measured using a cell counting kit-8 (Dojindo, Japan) according to the manufacturer’s protocol. Cell proliferation was expressed as the optical density.16,17

Coculture of VSMCs and macrophages: Twenty-four hours after extraction of macrophages, they were stimulated by TNF-α (40 ng/mL) and incubated for 6 days. Nonactivated macrophages were prepared by incubating for 24 hours after extraction. The macrophages were treated with mitomycin C for 30 minutes, and cocultured with VSMCs for 24 hours. The macrophages and VSMCs were then trypsinized and seeded

Figure 1. Effect of IMD-0354 treatment on neointimal formation after vascular injury. IMD-0354 (10 or 20 mg/kg/day or saline (nontreated)) was administered for 28 days after injury and the femoral arteries were excised. The sample sections were stained with EvG, and neointimal formation was evaluated. A: representative photographs of EvG staining. B: bar graphs show the I/M ratio. Data are mean ± SEM.
onto 96-well plates. Cell proliferation was measured after 24 hours of incubation.

**Statistical analysis:** Values are given as the mean ± SEM. Student’s *t* test was used to compare two groups. Groups were compared with Scheffé’s ANOVA. Differences were considered to be statistically significant at a value of $P < 0.05$.

**RESULTS**

**IMD-0354 suppressed neointimal formation:** In the nontreated group, injured arteries showed significantly thickened intima $(3.77 \pm 0.59, n = 8)$, while the IMD-0354 treated groups showed dose-dependently suppressed neointimal formation 4 weeks after arterial injury $(10 \text{ mg/kg/day}: 1.62 \pm 0.22, n = 10, P < 0.05; 20 \text{ mg/kg/day}: 1.05 \pm 0.28, n = 7, P < 0.05)$ (Figure 1). No neointimal formation was detected in arteries from sham operated mice (data not shown).

**IMD-0354 suppressed the expression of a proliferating factor:** To clarify the mechanism, we performed immunohistochemistry. Enhanced expression of PCNA was observed in the neointimal area of the nontreated injured arteries 2 weeks after injury, while IMD-0354 significantly suppressed PCNA expression (Figure 2). No expression of PCNA was detected in arteries from sham operated mice.

**Analysis of NF-κB activated cell types:** In order to detect what type of cells contributed to the inflammation after arterial injury, we performed immunofluorescence analysis using a confocal microscope. Nuclear translocation of p65 was detected in both α-SMA and CD11b positive cells, and p65 seemed more activated in α-SMA positive cells. IMD-0354 treatment suppressed nuclear translocation of p65 (Figure 3).

**IMD-0354 inhibited mRNA expression and protein production of proinflammatory factors:** We performed quantitative RT-PCR to determine the effects of IMD-0354 on proinflammatory factor mRNA expression. Arteries from sham-operated mice were used as the reference point (fold changes = 1). The expressions of COX-2, ICAM-1, IL-6, MCP-1, and MMP-3 mRNA expression were significantly inhibited by IMD-0354 treatment 1 week after injury. IMD-0354 treatment did not significantly suppress the expression of TNF-α and PAI-1. Arterial protein expression was also evaluated by Western blotting. PAI-1 expression was enhanced in arteries 1 week after injury, while IMD-0354 significantly suppressed the expression (Figure 4).

**IMD-0354 suppressed cytokine induced proliferation in vitro:** Initiation and maintenance of VSMC proliferation is a critical event in the pathogenesis of neointimal formation. IMD-0354
significantly inhibited VSMC proliferation induced by PDGF-BB, TNF-α, and IL-1β (Figure 5).

**IMD-0354 suppressed activated macrophage induced proliferation in vitro:** We hypothesized that the interaction between VSMCs and activated macrophages was an essential mechanism in VSMC proliferation in this model. The activated macrophages significantly inhibited VSMC proliferation induced by PDGF-BB, TNF-α, and IL-1β (Figure 5).

**Figure 4.** Effects of IMD-0354 on mRNA expressions and protein production of inflammatory factors. The femoral arteries were excised, homogenized and analyzed by quantitative RT-PCR (A) and Western blotting (B) 7 days after injury. A: the mRNA expressions of COX-2, ICAM-1, IL-6, MCP-1, and MMP-3 were attenuated by IMD-0354 treatment, while PAI-1 and TNF-α expressions were not significantly suppressed. The level of expression was normalized to that of 18s ribosomal RNA. We used arteries from sham-operated mice as the reference point (fold changes = 1). B: PAI-1 protein production was significantly suppressed by IMD-0354 treatment. Data are mean ± SEM.

**Figure 5.** Effects of IMD-0354 on VSMC proliferation triggered by cytokines. IMD-0354 prevented VSMC proliferation induced by PDGF-BB (A), TNF-α (B), and IL-1β (C). Bar graphs show absorbance which reflects cell viability. Data are mean ± SEM.
phages stimulated VSMC proliferation, while IMD-0354 negated this effect (Figure 6).

**Discussion**

This is the first report to provide evidence of the beneficial effects of the selective IKK-β inhibitor IMD-0354 on the development of neointimal formation after arterial injury. This was caused by suppressing inflammatory factor expression and VSMC proliferation. The results obtained in this study were as follows; 1. The novel IKK-β inhibitor IMD-0354 prevented neointimal formation after wire-mediated arterial injury, 2. IMD-0354 inhibited the inflammatory response in vivo, and 3. IMD-0354 attenuated VSMC proliferation induced by cytokines and activated macrophages.

NF-κB is deeply involved in neointimal formation after arterial injury, and has been investigated as a novel therapeutic target to prevent restenosis. Previously, our group reported on the effectiveness of NF-κB decoy oligonucleotide transfection on neointimal formation and the clinical safety of the approach in humans. In this study, we used IMD-0354, which is a synthesized agent that inhibits IKK-β activity only under inflammatory conditions. IMD-0354 inhibited 98.5% of NF-κB activity, but did not influence other kinases, proteases or proteasome-related immune responses. The safety of IMD-0354 has been demonstrated by several previous reports. This study revealed IMD-0354 dose-dependently attenuated neointimal formation after wire-mediated arterial injury in mice. Thus, IMD-0354 has the potential to prevent restenosis in humans with fewer adverse effects compared to conservative therapies.

The central feature of neointimal formation is increased VSMC proliferation and the subsequent formation of extracellular matrix deposition by VSMCs. These events are thought to be triggered by vascular inflammation. Mechanical vascular injury induced NF-κB activation, which leads to the expression of several proinflammatory genes in VSMCs. IMD-0354 showed anti-inflammatory activity by inhibiting IL-6, Cox-2, MCP-1, MMP-3, and ICAM-1 mRNA expression from injured arteries. Among these, MCP-1 has been reported to play essential and multiple roles in vascular remodeling, such as the recruitment of inflammatory cells or VSMC proliferation/migration. Therefore, the suppression of MCP-1 expression by IMD-0354 is an effective way to prevent neointimal formation. Furthermore, COX-2 down-regulation may contribute to the prevention of neointimal thickening as we previously reported. Thus, IMD-0354 has beneficial anti-inflammatory effects on wire-injured arteries. Additionally, IMD-0354 treatment inhibited PAI-1 protein expression without affecting PAI-1 mRNA expression. Takaoka, et al reported phenotypic changes in perivascular adipose tissue after endovascular injury and that the injury caused enhanced PAI-1 mRNA expression in fat tissue. As PAI-1 down-regulation on neointimal formation is highly controversial, further detailed study is required.

The antiproliferative effect of IMD-0354 was also verified. Treatment with IMD-0354 diminished the number of PCNA-positive proliferating cells in neointimal lesions in mice 2 weeks after arterial injury. Furthermore, IMD-0354 inhibited in vitro mouse-derived VSMC proliferation induced by PDGF-BB, TNF-α, and IL-1β. Previous reports have suggested that there are fewer inflammatory cells in the neointima, and that bone marrow-derived cells in the neointima lesion mainly differentiate into macrophage lineages. Thus, the main role of these cells in the development of a neointimal lesion is to initiate inflammation and induce VSMC proliferation. Kosuge, et al indicated that activated T cells induced VSMC proliferation, which might contribute to neointimal formation of atherosclerosis. In this study, we extracted macrophages from mice and induced their activation by TNF-α stimulation. Next, we cocultured the macrophages with murine VSMCs. Cross-interaction between macrophages and VSMCs was found and activated macrophages significantly induced VSMC proliferation. This in vitro experimental model can be useful to study the specific roles of macrophages in the development of neointimal lesions. Interestingly, IMD-0354 effectively attenuated proliferation. Along with our immunofluorescence results that indicated VSMCs primarily contributed to NF-κB activation, IMD-0354 may affect VSMCs rather than macrophages.

VSMC apoptosis is known to be involved in the pathological mechanism of neointimal formation. It has been shown that apoptotic VSMCs amplify vascular inflammation by releasing proinflammatory cytokines. The inhibition of medial VSMC apoptosis prevents neointimal formation after vascular injury. Several reports demonstrated that IMD-0354 induced apoptosis of cancer related cells, therefore, this drug may have the same effects on VSMC. Further study will be needed to clarify the role of IMD-0354 on the apoptotic effects in the progression of vascular remodeling.

In conclusion, IMD-0354 had anti-inflammatory and antiproliferative effects on VSMCs, which lead to the suppression of neointimal formation after wire-mediated arterial injury. Thus, this drug may effectively prevent restenosis after coronary intervention and other cardiovascular diseases.

**Acknowledgments**

We thank Ms. Noriko Tamura and Ms. Yasuko Matsuda for their excellent assistance.

**References**

7. Suzuki J, Ito H, Gotoh R, Morishita R, Egashira K, Isobe M. Initial clinical cases of the use a NF-kB decoy at the site of coronary