

Original Article

MMP-9 Inhibition by ACE Inhibitor Reduces Oxidized LDL-Mediated Foam-Cell Formation

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Aim: Angiotensin-converting enzyme inhibitors (ACEIs) have been shown to block matrix metalloproteinase (MMP)-9 activity, which plays a role in atherogenesis. MMP-9 activity of macrophages is increased during foam cell formation. To investigate the contribution of ACEIs to foam cell formation, we studied the effects of an ACEI, imidaprilat, on THP-1 macrophages and the underlying molecular mechanisms *in vitro*.

Methods and Results: Pre-treatment of THP-1 macrophages with imidaprilat (100 nmol/L, 4 hours) significantly decreased foam cell formation induced by oxidized LDL (OxLDL). Imidaprilat reduced the protein level of MMP-9 in THP-1 macrophages and attenuated OxLDL-induced MMP-9 activity in the culture supernatants. Indeed, pretreatment of THP-1 macrophages with an MMP-2/9 inhibitor (20 μ mol/L, 4 hours) attenuated OxLDL-induced foam-cell formation. Imidaprilat or the MMP-2/9 inhibitor blocked OxLDL-induced expressions of LOX-1 and scavenger receptor-A (SR-A), but not that of CD36, in THP-1 macrophages. In addition, OxLDL-induced activation of p38 mitogen-activated protein kinase (MAPK) and ERK, but not JNK, was blunted by imidaprilat or the MMP-2/9 inhibitor. Finally, siRNA against MMP-9 inhibited foam cell formation as well as lipid accumulation in THP-1 macrophages.

Conclusion: These findings suggest that imidaprilat reduces OxLDL-triggered foam-cell formation in THP-1 macrophages via modulation of MMP-9 activity and may indicate a novel anti-inflammatory mechanism of imidaprilat in atherogenesis.

J Atheroscler Thromb, 2010; 17:97-105.

Key words; ACE inhibitor, MMP-9, Oxidized LDL, Scavenger receptors, Foam cell formation

Introduction

It is evident that dyslipidemia and the renin angiotensin system play a crucial role in atherogenesis¹. Angiotensin-converting enzyme inhibitors (ACEIs) exert beneficial effects on vascular function and protect against atherosclerosis and cardiovascular events^{2,3}.

One of the initial events during atherosclerosis development is the appearance of lipid-loaded foam cells derived from macrophages in the arterial intima.

During this process, oxidized LDL (OxLDL), generated by modification of LDL with oxidative stress, is taken up by macrophages⁴. Thus, inhibition of OxLDL-induced macrophage foam-cell formation may contribute to the prevention of atherosclerosis⁵.

ACEIs have been shown to attenuate the development of atherosclerosis in hypercholesterolemic animal models. For example, ramipril reduced OxLDL uptake of macrophages in ApoE knockout mice⁶, and treatment with ACEIs inhibited LDL oxidation^{7,8}. These atheroprotective activities of ACEIs potentially contribute to their beneficial effects, which have been shown in several clinical trials.

Recent reports point to novel angiotensin II-independent mechanisms in the anti-atherosclerotic and anti-inflammatory actions of ACE inhibitors⁹⁻¹¹. We recently reported that ACEIs had effects on zinc

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Received: March 5, 2009

Accepted for publication: July 3, 2009

metabolism, which modulates such inflammatory processes as cell adhesion¹²). Moreover, they are able to bind directly to matrix metalloproteinase (MMP)-9 and suppress its activity^{13, 14}), leading to cardiovascular protection^{15, 16}).

MMPs are endopeptidases capable of cleaving components of the extracellular matrix, and their expressions have been documented in a variety of cell types, including smooth muscle cells, endothelial cells, and macrophages. Furthermore, several members of the MMP family are found in atherosclerotic plaques^{17, 18} and arterial aneurysms¹⁹). MMP-9, also known as gelatinase B, processes multiple extracellular substrates, including denatured collagen I, fibronectin, and laminin. OxLDL upregulates MMP-9 expression in THP-1 macrophages by activating the MEK/ERK signaling pathway^{20, 21}), while a recent report noted that deletion of MMP-9 reduced the growth of atherosclerotic lesions in ApoE knockout mice²²). MMP-9 inhibition decreases macrophage migration, plaque rupture, smooth muscle cell proliferation and angiogenesis, but the causative role of MMP-9 in OxLDL-induced macrophage foam-cell formation is unknown.

In the present study, we investigated the potential role of imidaprilat, an active metabolite of imidapril, in macrophage foam-cell formation, as well as the possible association of MMP-9 in that process.

Material and Methods

Reagents and Antibodies

Imidaprilat was kindly provided by Mitsubishi Tanabe Pharma Corporation, Osaka, Japan. The reagents used in the present study were phorbol 12-myristate 13-acetate (PMA), Cholesterol kit (Denka Seiken), Triglyceride E test reagent (Wako), RPMI 1640 and Dulbecco's phosphate-buffered saline (DPBS) (Sigma). The antibodies were anti-p38 mitogen-activated protein kinase (MAPK) and anti-p38 MAPK (Cell Signaling), anti-MMP-9 (Chemicon International), anti-CD36, anti-pERK, anti-ERK1, anti- α tubulin (Santa Cruz), and anti-LOX-1 and anti-scavenger receptor A (SR-A) (R&D Systems). We also utilized an MMP-2/9 inhibitor (Calbiochem).

Cell Culture

THP-1 cells, a human monocytic cell line, were obtained from the American Type Culture Collection (ATCC) and European Collection of Cell Cultures (ECACC). THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin, 100 μ g/mL strep-

tomycin, and 2 mmol/L L-glutamine in a humidified atmosphere of 5% CO₂ at 37°C. To examine cell viability, THP-1 cells were stained with DAPI solution after incubation with imidaprilat, the MMP-2/9 inhibitor, or OxLDL.

Preparation of LDL

Human LDL (1.019 to 1.063 g/mL) was isolated from the plasma of healthy human subjects by sequential ultracentrifugation at 4°C, and then oxidized by exposure to CuSO₄ (10 μ mol/L) in DPBS at 37°C for 24 hours. OxLDL was extensively dialyzed against 0.24 mmol/L EDTA-saline. The level of endotoxin was measured using an E-Toxate kit (Wako) and found to be consistently less than 0.03 EU/mL (lowest detection limit).

Immunoblotting

Lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then Western blotting was carried out with the antibodies as described above.

Oil Red O Staining

THP-1 macrophages were seeded into multi-well slides (Nunc) at a concentration of 2×10^5 cells per well. The cells were washed 3 times with DPBS, fixed with formaldehyde, and stained with oil red O. Lipid accumulation was observed under a microscope.

Cellular Lipid Analysis

To determine intracellular lipid contents, THP-1 macrophages were washed with DPBS and then removed from the culture plates, after which intracellular lipids were extracted using isopropanol. Triglyceride (TG) and total cholesterol (TC) were measured using an enzymatic method.

MMP Activity Measurement

MMP activity in THP-1 macrophages was measured using two different methods.

a) Gelatin Zymography

Gelatin zymography of THP-1 macrophages was conditioned as previously described¹⁷). Cells were seeded at 2×10^6 cells/mL in medium containing PMA at 200 nmol/L for 3 days, and the medium was then changed to fresh 1% FCS medium with or without imidaprilat, the MMP-2/9 inhibitor for 4 hours, followed by stimulation with OxLDL (100 μ g/mL) for 24 hours. Supernatants were subjected to SDS-PAGE in 6% (w/v) polyacrylamide gels that had been copolymerized with 0.2% gelatin. After electrophoresis, the

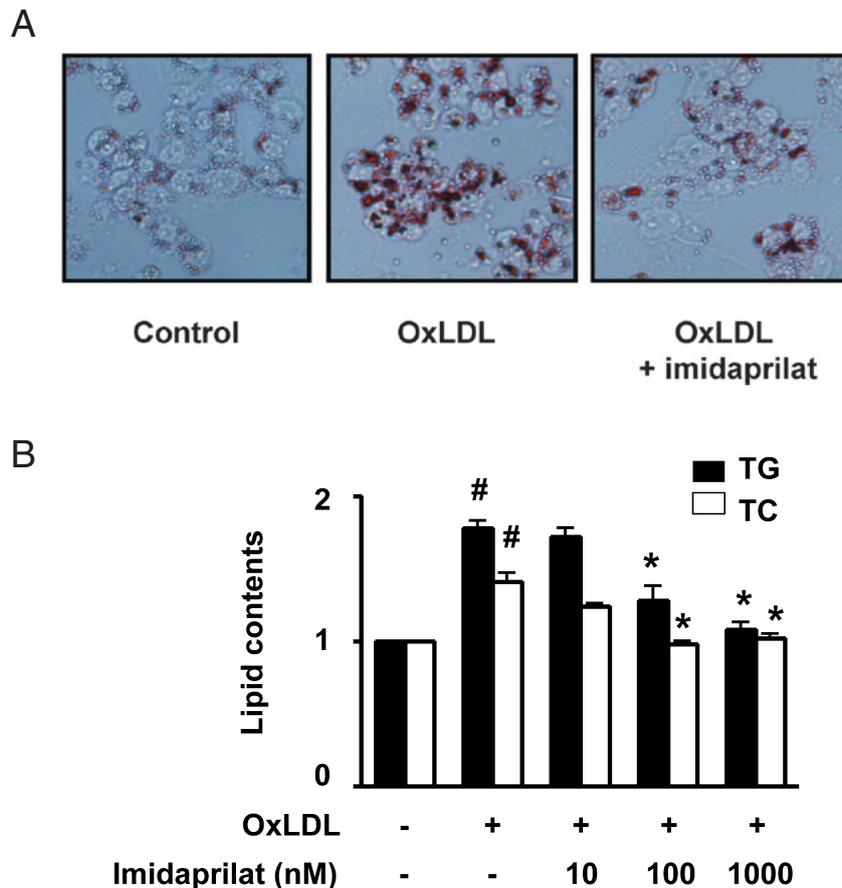


Fig. 1. Imidaprilat reduced foam-cell formation in THP-1 macrophages.

(A) THP-1 cells were incubated in the presence or absence of imidaprilat (100 nmol/L) for 4 hours, followed by incubation with OxLDL (100 μ g/mL) for 48 hours. Photos show representative oil red O staining results obtained in 3 separate experiments. (B) Bar graphs show lipid contents in THP-1 macrophages ($n=4$). THP-1 macrophages were incubated in the presence of the indicated concentrations of imidaprilat for the indicated periods, followed by stimulation with OxLDL (100 μ g/mL) for 48 hours prior to the assay. Lipid content was measured as described in Methods. Data are representative of 3 independent experiments. # $p < 0.01$ vs cont. * $p < 0.01$ vs OxLDL.

gels were stained with Coomassie Brilliant Blue (Fluka AG). Proteolytic activity in a particular gel location yielded a clear band against the blue background of the stained gelatin.

b) MMP-9 Quenched Substrate Assays

The activity of MMP-9 in conditioned supernatants was determined using an EnzoLyte MMP-9 fluorometric assay kit (AnaSpec, Inc.). Briefly, conditioned supernatants were incubated with MMP-specific peptide substrates, based on the instructions included with the kit. Cleavage of substrates by MMPs removed the quenching effect, resulting in increased fluorescence with excitation at 360 nm and emission at 460 nm.

MMP-9 siRNA Experiment

MMP-9 siRNA was purchased from Santa Cruz. siRNA against β -galactosidase (Lac Z) was used as a control. THP-1 macrophages were seeded at 7.5×10^5 in each well of a C-6 plate and transfected with 5 μ L Lipofectin 2000 (Invitrogen) and the control siRNA or MMP-9 siRNA in 1 mL of OptiMax (final concentration of siRNA 100 nmol/L). Thirty-six hours after transfection, the cells were co-incubated with OxLDL (100 μ g/mL) for 48 hours.

Statistical Analysis

Results are presented as the mean \pm SEM. Data were analyzed using ANOVA with Tukey's post-hoc analysis. A p value 0.05 was considered significant.

Results

Imidaprilat Inhibits OxLDL-Induced Foam-Cell Formation by THP-1 Macrophages

We investigated the effects of imidaprilat on macrophage foam-cell formation. First, THP-1 cells were incubated in the presence of PMA at a concentration of 200 nmol/L for 3 days. After the medium was replaced with 1% FCS/RPMI medium with or without imidaprilat for 4 hours, OxLDL (100 μ g/mL) was added and incubated for an additional 48 hours. As shown in **Fig. 1A**, OxLDL induces intracellular lipid accumulation (red dye) in THP-1 macrophages. In contrast, co-treatment with imidaprilat reduced intracellular lipid accumulation. To quantitate the observed lipid accumulation, intracellular TG and TC were measured. OxLDL-mediated TG and TC accumulation in THP-1 macrophages was inhibited by imidaprilat treatment (**Fig. 1B**) and the inhibitory effect of imidaprilat was observed up to 100 nmol/L ($p < 0.01$); therefore, we incubated THP-1 cells with imidaprilat at 100 nmol/L in the subsequent experiments.

Imidaprilat Decreases OxLDL-Triggered MMP-9 Activity in Culture Supernatant

Next we focused on the effects of imidaprilat on MMP-9 activity in THP-1 macrophages, since OxLDL has been reported to increase MMP-9 activity and ACEIs directly modulate that activity^{13, 14}. In gelatin zymography analysis, imidaprilat reduced MMP-9 activity in the culture supernatants (**Fig. 2A**). Further, imidaprilat or the MMP-2/9 inhibitor each abolished OxLDL-induced elevation of MMP-9 activity (**Fig. 2B**). The results of the quenched substrate assay revealed that OxLDL increased MMP-9 activity in the culture supernatant, which was blunted by imidaprilat (**Fig. 2C**). Further, OxLDL-induced MMP-9 protein expression in THP-1 macrophages was slightly reduced by imidaprilat (**Fig. 2D**).

MMP-2/9 Inhibitor Attenuates OxLDL-Induced Foam-Cell Formation

To directly link MMP-9 activity with foam-cell formation, the effects of an MMP-2/9 inhibitor were examined. As shown in **Fig. 3A**, treatment with the MMP-2/9 inhibitor markedly suppressed foam-cell formation induced by OxLDL. Further, TG and TC contents increased after OxLDL treatment were diminished by MMP-2/9 inhibitor treatment (**Fig. 3B**).

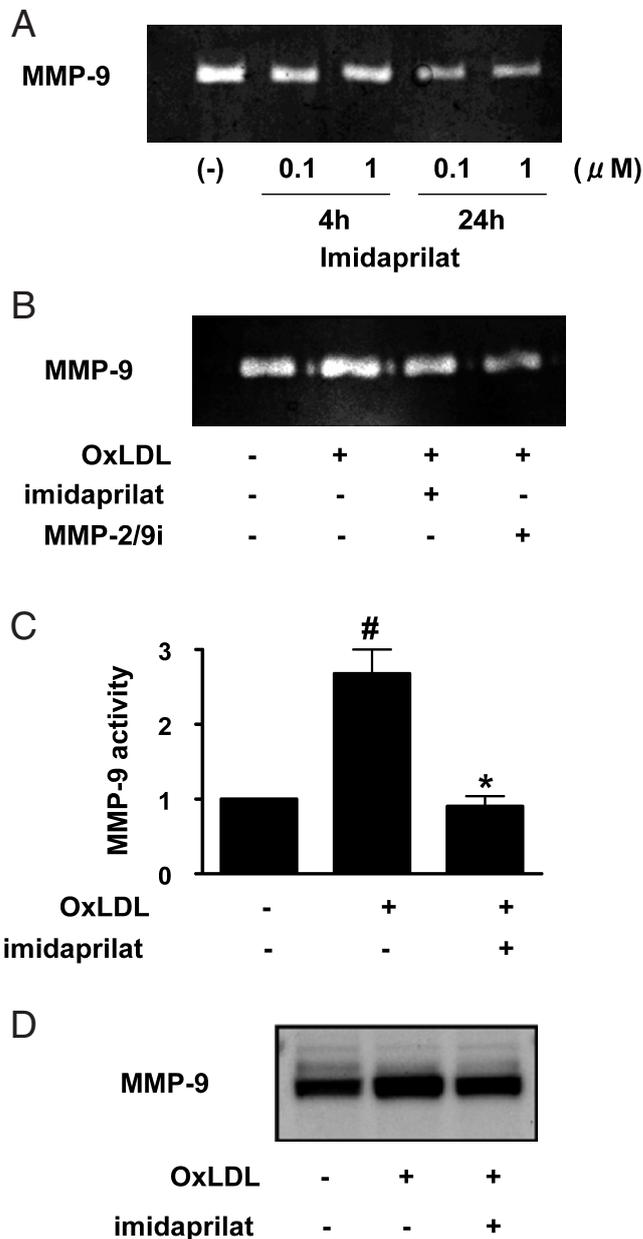


Fig. 2. Imidaprilat attenuated OxLDL-induced MMP-9 activity.

(A) THP-1 macrophages were incubated with imidaprilat (100 nmol/L or 1,000 nmol/L) for 4 or 24 hours. MMP-9 activity in culture supernatants decreased in a dose- and time-dependent manner. (B) THP-1 macrophages were treated with imidaprilat (100 nmol/L) or the MMP-2/9 inhibitor (20 μ mol/L), and then stimulated with OxLDL (100 μ g/mL). Culture supernatants were collected after 24 hours for determination of MMP-9 activity by gelatin zymography. (C) THP-1 macrophages were preincubated for 4 hours in the presence or absence of imidaprilat (100 nmol/L) followed by incubation with 100 μ g/mL OxLDL. MMP-9 activity in the culture supernatants was analyzed by using an MMP-9 fluorometric assay kit. [#] $p < 0.05$ vs cont. ^{*} $p < 0.05$ vs OxLDL. (D) Imidaprilat reduced OxLDL-induced MMP-9 expression in THP-1 macrophages.

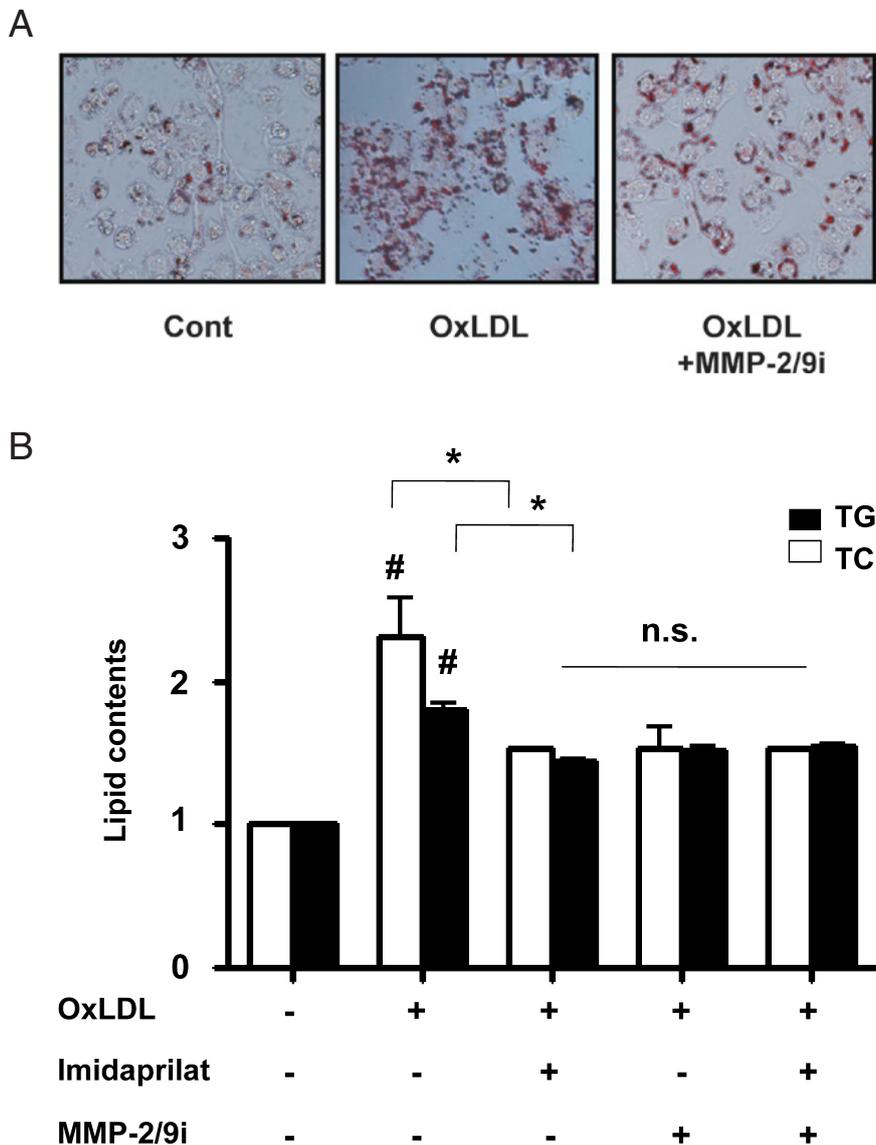


Fig. 3. MMP-2/9 inhibitor reduced foam-cell formation in THP-1 macrophages.

(A) THP-1 cells were incubated in the presence or absence of the MMP-2/9 inhibitor (20 $\mu\text{mol/L}$) for 4 hours, followed by incubation with OxLDL (100 $\mu\text{g/mL}$) for 48 hours. Photos show representative oil red O staining results obtained in 3 separate experiments. (B) Bar graphs show cellular TG and TC contents in THP-1 macrophages ($n=4$). THP-1 macrophages were incubated in the presence of imidaprilat (100 nmol/L) alone or the MMP-2/9 inhibitor (20 $\mu\text{mol/L}$) alone or a combination of both for 4 hours, followed by stimulation with OxLDL (100 $\mu\text{g/mL}$) for 48 hours. Lipid content was measured out as described in Methods. Data are representative of 3 experiments. # $p < 0.05$ vs cont. * $p < 0.05$ vs OxLDL.

Imidaprilat or MMP-2/9 Inhibitor Decrease OxLDL-Induced LOX-1 and SR-A Expression in THP-1 Macrophages

The effects of imidaprilat or the MMP-2/9 inhibitor on the expression of scavenger receptors during foam-cell formation were also examined. As shown in **Fig. 4A, 4B**, imidaprilat or MMP-2/9 inhibitor

decreased OxLDL-triggered LOX-1 and SR-A expression in THP-1 macrophages. In contrast, the expression level of another scavenger receptor, CD36, was not changed by imidaprilat, MMP-2/9 inhibitor, or OxLDL treatment.

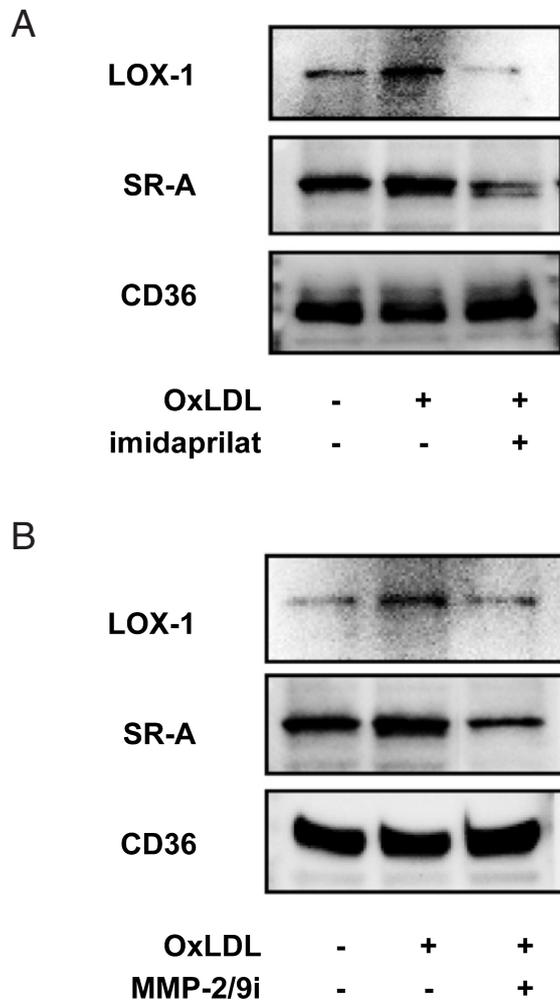


Fig. 4. Imidaprilat (A) or MMP-2/9 inhibitor (B) decreased OxLDL-induced LOX-1 and SR-A expression in THP-1 macrophages.

THP-1 cells were incubated in the presence or absence of imidaprilat (100 nmol/L) or MMP-2/9 inhibitor (20 μ mol/L) for 4 hours, followed by stimulation with OxLDL (100 μ g/mL) for 48 hours prior to the assay. LOX-1, SR-A and CD36 were detected with immunoblotting (IB). Data representative of 3 independent experiments.

Imidaprilat or MMP-2/9 Inhibitor Inhibit OxLDL-Induced MAP Kinase Activation in THP-1 Macrophages

We also examined the signaling pathways involved in the effects of imidaprilat or the MMP-2/9 inhibitor on foam-cell formation. THP-1 macrophages were pretreated for 4 hours with imidaprilat (100 nmol/L) or the MMP-2/9 inhibitor (20 μ mol/L) before exposure to OxLDL. As shown in **Fig. 5A**, OxLDL increased p38 MAPK and ERK phosphorylation, which was inhibited by imidaprilat (**Fig. 5A**) or

the MMP-2/9 inhibitor (**Fig. 5B**). Activation of JNK by OxLDL was not significantly detected under our assay conditions (data not shown).

siRNA Against MMP-9 Decreases OxLDL-Induced Foam-Cell Formation in THP-1 Macrophages

To confirm the causative role of MMP-9 in OxLDL-induced foam-cell formation, we introduced siRNA against MMP-9 into THP-1 macrophages. Transfection of THP-1 macrophages with MMP-9 siRNA successfully reduced MMP-9 expression (**Fig. 6A**). Oil red O staining revealed a reduction of lipid accumulation in THP-1 macrophages transfected with MMP-9 siRNA as compared to those transfected with control siRNA following OxLDL treatment (**Fig. 6B**). Moreover, the expression levels of LOX-1 and SR-A were attenuated in THP-1 macrophages transfected with MMP-9 siRNA as compared to those with control siRNA (**Fig. 6C**).

Discussion

The present study demonstrated that imidaprilat, an ACEI that significantly reduces OxLDL-triggered foam-cell formation in THP-1 macrophages, is involved in inhibition of MMP-9.

OxLDL has been shown to increase the expression of ACE²³ and upregulate the angiotensin II type 1 receptor in cultured human coronary artery endothelial cells²⁴; however, it is interesting that ACE expression in THP-1 macrophages was not significantly changed by OxLDL in our assay conditions (data not shown). Moreover, OxLDL treatment did not increase ACE activity in the culture supernatants (data not shown). These results suggest that the activities of imidaprilat in modulating foam-cell formation may not be related to its effect on ACE.

Collagen, as a component of the extracellular matrix, has been linked to atherosclerotic plaque formation and stability. The activity of MMP-9, a zinc-dependent endopeptidase, is markedly increased after myocardial infarction and rupture of abdominal aortic aneurysms^{19, 25}; therefore, MMP secretion from macrophages or smooth muscle cells could contribute to plaque rupture. OxLDL upregulates MMP-9 expression in monocyte-derived macrophages and endothelial cells^{21, 26}. Further, a previous study using MMP-9 knockout mice demonstrated its importance in atherosclerosis²².

ACE, like MMP-9, is also a zinc-dependent endopeptidase. In previous reports, ACEIs directly inhibited MMP-9 activity in tissue extracts by stabilizing hydrophobic interactions in its active domain^{13, 14}.

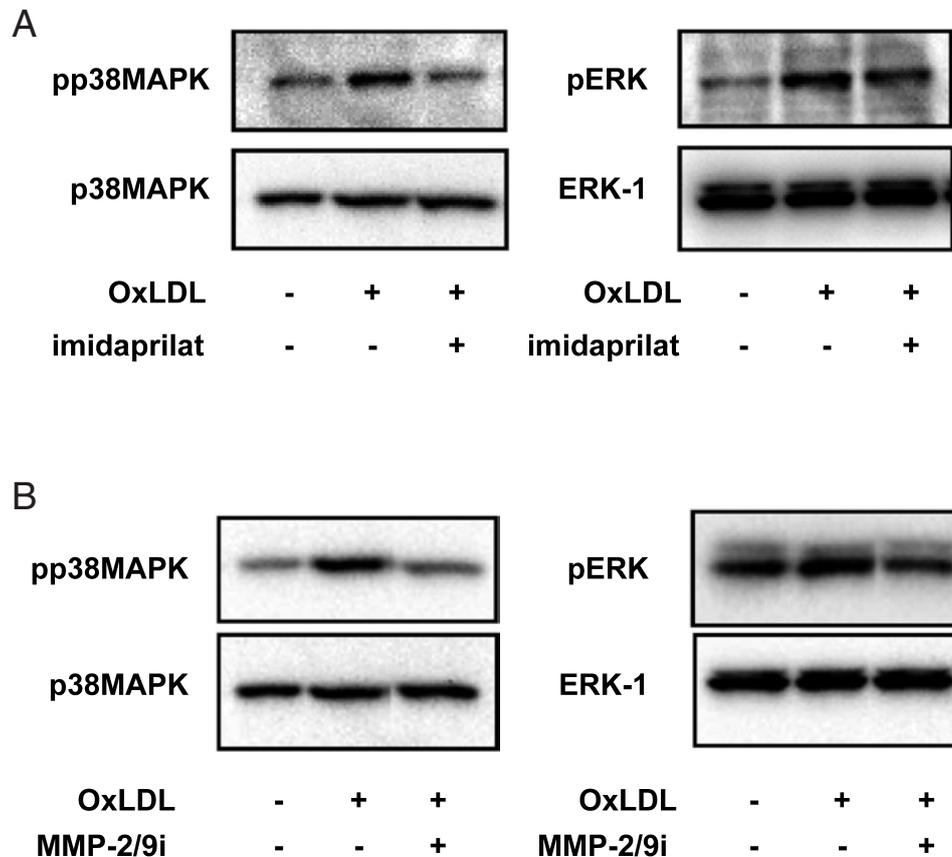


Fig. 5. Imidaprilat or MMP-2/9 inhibitor inhibited MAP kinase activation.

THP-1 macrophages were incubated for 4 hours with imidaprilat (100 nmol/L) or the vehicle, and then stimulated with OxLDL (100 μ g/mL) for 48 hours. Imidaprilat (A) or MMP-2/9 inhibitor (B) reduced the phosphorylation of p38 MAPK and ERK induced by OxLDL stimulation (100 μ g/mL, 48 hours). Three independent experiments were performed, with representative results shown.

In line with those findings, we confirmed that an increase in MMP-9 activation in THP-1 macrophages was correlated with foam-cell formation, which was attenuated by imidaprilat treatment. Imidaprilat treatment alone diminished MMP-9 activity in culture supernatant *in vitro*. Furthermore, OxLDL-induced foam cell formation was diminished by treatment with the MMP-2/9 inhibitor. There is no additional effect of MMP-2/9 inhibitor on the inhibiting effect of imidaprilat. To identify the contribution of MMP-9 to the anti-inflammatory properties of imidaprilat, we conducted additional experiments using siRNA against MMP-9 and found that gene silencing of MMP-9 directly leads to successful inhibition of OxLDL-induced foam cell formation, suggesting the importance of MMP-9 in foam-cell formation. Since no MMP-2 activity was detected in THP-1 macrophage culture supernatants under our assay conditions, MMP-9 may play a primary role in macrophage foam-

cell formation induced by OxLDL.

As previously reported, OxLDL activates MAP kinases in smooth muscle cells and macrophages^{27, 28}, and that activation is critical in atherosclerosis. In the present study, we observed a reduction of ERK phosphorylation and p38 MAPK following imidaprilat treatment or the MMP-2/9 inhibitor, which suggests the importance of MAP kinases in the atheroprotective action of MMP-9 inhibition.

During the differentiation of monocytes into macrophages, scavenger receptor expression is upregulated, which is one of the key events in the process of atherosclerosis. Scavenger receptors excessively take up OxLDL, leading to their conversion to foam cells²⁹. LOX-1 is a scavenger receptor that is highly expressed in atherosclerotic lesions^{30, 31}. In line with these results, we found that expression levels of LOX-1 and SR-A were upregulated by OxLDL treatment, which was reduced by imidaprilat or MMP-2/9 inhibitor treat-

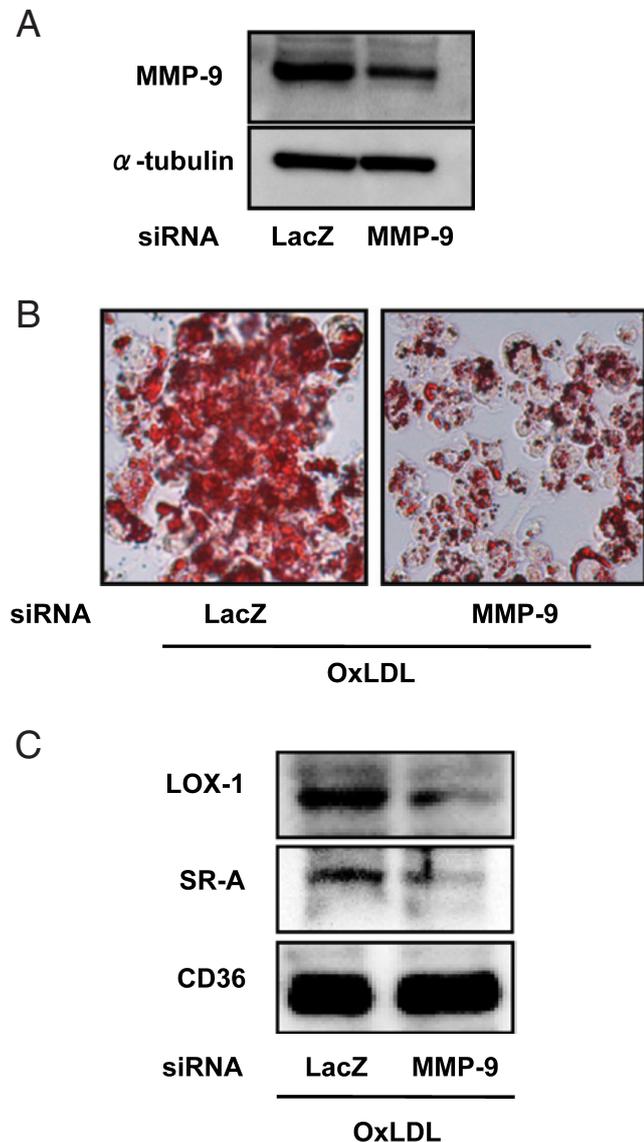


Fig. 6. siRNA against MMP-9 decreased OxLDL-induced foam-cell formation in THP-1 macrophages.

(A) THP-1 macrophages were transfected with MMP-9 siRNA or control siRNA as described in Methods. Twenty-four hours after transfection, the expression of MMP-9 was detected with immunoblotting (blots are representative of 3 separate experiments). (B) After transfection of THP-1 macrophages with siRNA for MMP-9 and LacZ, THP-1 macrophages were incubated with OxLDL (100 μ g/mL, 48 hours) and oil red O staining was performed. (C) LOX-1 and SR-A expression induced by OxLDL (100 μ g/mL, 48 hours) were detected in THP-1 macrophages after siRNA transfection by immunoblotting.

ment. Since the expression level of LOX-1 is regulated through MAP kinases³², imidaprilat or MMP-2/9 inhibitor-mediated modulation of LOX-1 may involve these pathways.

As previously reported³³, another ACEI, captopril, also inhibits MMP-9 activity. We also confirmed that captopril decreased OxLDL-mediated TG accumulation in THP-1 macrophages (data not shown). Thus, our observation using imidaprilat may be a class effect of ACEIs. From a pathophysiological point of view, our data provide a new mechanistic explanation and a molecular basis for the potential clinical use of ACEI or MMP inhibitors to prevent OxLDL-induced foam-cell formation.

In conclusion, our results showed that an ACEI, imidaprilat, inhibited OxLDL-triggered foam-cell formation. The underlying mechanisms seem to involve the modulation of MMP-9 activity in addition to effects on ACE or reactive oxygen species (ROS) inhibition.

Acknowledgement

This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan.

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