Midkine Promotes Atherosclerotic Plaque Formation Through Its Pro-Inflammatory, Angiogenic and Anti-Apoptotic Functions in Apolipoprotein E-Knockout Mice

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Background: A recent study suggested that midkine (MK), a heparin-binding growth factor, is associated with atherosclerosis progression in patients with artery disease. It has previously been reported that MK plays a critical role in neointima formation in a restenosis model, whereas the role of MK in the development of atherosclerosis has not been investigated. The present study assessed the effect of MK administration on the process of atherosclerotic plaque formation in apolipoprotein E-knockout (ApoE−/−) mice.

Methods and Results: Using an osmotic pump, human recombinant MK protein was intraperitoneally administered for 12 weeks in C57BL/6 ApoE−/− (ApoE−/−-MK) and ApoE+/+ mice fed a high-fat diet. Saline was administered to the control groups of ApoE−/− (ApoE−/−-saline) and ApoE+/+ mice. The atheroerotic lesion areas in longitudinal aortic sections were significantly larger in ApoE−/−-MK mice than in ApoE−/−-saline mice. The aortic mRNA levels of pro-inflammatory and angiogenic factors, and the percentage of macrophages in aortic root lesions, were significantly higher in ApoE−/−-MK mice than in ApoE−/−-saline mice, whereas the percentage of apoptotic cells was significantly lower in ApoE−/−-MK mice than in ApoE−/−-saline mice.

Conclusions: The systemic administration of MK in ApoE−/− mice promoted atherosclerotic plaque formation through pro-inflammatory, angiogenic, and anti-apoptotic effects. MK may serve as a potential therapeutic target for the prevention of atherosclerosis under atherogenic conditions.

Key Words: Angiogenesis; Apoptosis; Atherosclerosis; Inflammation; Midkine
studies have shown that obesity induces chronic inflammation within adipose tissue, leading to metabolic abnormalities and inflammation in distant tissues. MK expression is increased in the adipose tissue of obese mice, and, more importantly, serum MK levels are significantly increased in overweight/obese human subjects than in controls, suggesting an association between MK and obesity. Moreover, MK is present in the thickened intima of fatty streaks containing VSMCs, ECs, and inflammatory cells, as well as in advanced atherosclerotic regions in human atheromatous plaques. Serum MK levels were significantly higher in patients with severe peripheral artery disease than in healthy controls. Remarkably, a recently published study demonstrated that MK could be used as a coronary atherosclerosis diagnostic tool in humans to predict the presence of anatomically significant coronary artery disease. Taken together, these findings suggest that MK mediates atherosclerotic plaque formation under atherogenic conditions.

The central role of MK in atherosclerosis has not yet been investigated. Here, we hypothesized that MK promotes atherosclerotic plaque formation via the enhancement of VSMC migration and macrophage migration, as well as the inhibition of macrophage apoptosis in atheromatous plaques. In the present study, we used apolipoprotein E-knockout (ApoE−/−) mice to investigate the chronic effect of continuous systemic administration of MK on spontaneously developing atherosclerosis without mechanical injury.

### Methods

#### Mouse Models of Atherosclerosis and Study Protocol

All animal experiments were performed in accordance with the regulations of the National Institute of Health and approved by the Animal Care and Use Committee of Nagoya University. ApoE−/− mice were supplied by the Department of Cardiology, Nagoya University Graduate School of Medicine, Nagoya, Japan. Eight-week-old male wild-type C57BL/6 (ApoE+/+) and ApoE−/− mice with a C57BL/6 genetic background were fed a high-fat diet (24.2% protein, 13.6% fat, 46.3% calories; CLEA JAPAN, Japan) for 12 weeks (Figure 1A). Either saline or human recombinant MK protein in saline (1.0 mg/mL) was continuously infused into ApoE−/− and ApoE−/− mice using an osmotic pump (Alzet, DURECT Corporation, Cupertino, CA, USA), as shown in Figure 1B. The MK protein was purified from yeast by anion exchange chromatography and affinity chromatography on a heparin column, as previously reported. Surgery to implant the osmotic pumps was performed under anesthesia with pentobarbital (100 mg/kg, intraperitoneally). The pumps infused a total of 90 μL continuously over a period of 2 weeks. The pumps were implanted under the abdominal skin and were exchanged every 2 weeks for a total of 12 weeks.

#### Serum Cholesterol and MK Levels, and Lipoprotein Profile Measurements

The serum cholesterol levels and lipoprotein profile were determined by using kits for clinical use, which were supplied by SRL Inc. (Tokyo, Japan). The serum MK levels were evaluated using an ELISA kit for MK (Wuhan USCYN Business Co., Ltd., Hubei, China).

#### Lesion Assessment

After anesthesia with pentobarbital, perfusion fixation was performed by infusing 4% paraformaldehyde through the left ventricular apex, and the entire aorta, from the ascending aorta to the abdominal aorta and up to the bifurcation of the common iliac arteries, was removed and fixed in 4% paraformaldehyde for 12–16 h at 4°C. Adventitial fat and extraneous tissue were dissected, and the aorta was split open longitudinally and stained with Sudan IV to visualize the extent of atherosclerosis. The percentage of the aortic surface covered by atheroma was quantified using Image Pro 6.1 (Media Cybernetics, Inc., MD, USA). The stained areas are presented as the percentage of the total plaque area.

#### Immunohistochemistry

After the mice were euthanized and perfused with ice-cold phosphate-buffered saline (PBS), perfusion fixation was performed prior to histological and immunohistochemical analyses, by infusing 4% paraformaldehyde through the
left ventricular apex. Serial 6-μm-thick paraffin sections of the aortic root were prepared as described previously.29 dewaxed in xylene, rehydrated in ethanol, and washed in PBS. The sections were then incubated in 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. For immunohistological analysis of macrophages, the sections were incubated for 12–16 h at 4°C with a polyclonal rabbit anti-mouse α-smooth muscle actin antibody (BD Biosciences, Co, USA) and counterstained with hematoxylin. The total cell population was estimated by counting the DAPI-stained nuclei (a total of 400–1,000 nuclei were counted per specimen), and the ratio of TUNEL-positive cells to DAPI-stained nuclei was calculated.

### Quantitative Real-Time Polymerase Chain Reaction
Total RNA was extracted from the entire aorta and was purified using an RNeasy Fibrous Tissue Mini Kit (QIAGEN Inc., CA, USA). Briefly, RNA was first converted to cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems Inc., CA, USA). Then, quantitative polymerase chain reaction (PCR) was performed using an ABI 7700 instrument (Applied Biosystems Inc.) with fluorescent TaqMan probes.34 The primers and assay ID information for quantitative real-time PCR are listed in Table B.

### Statistical Analyses
All the values are presented as mean±standard error of the mean (S.E.M.). Multi-group comparisons were analyzed by analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. Direct 2-group comparisons were performed using the 2-sided Student’s t-test. Differences with a P<0.05 were considered significant.

### Results
**MK Increases Atherosclerosis**
Serum MK levels increased significantly after MK administration in both ApoE+/- and ApoE−/− mice (Table A). MK increased the area of atherosclerotic lesions in ApoE−/− mice in en face preparations of the aortic root, as deter-

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**Table.** (A) Serum Cholesterol Levels, Lipoprotein Profiles, and MK Levels at the Time of Mouse Sacrifice (20 Weeks Old), (B) List of Primers and Assay ID Information for Quantitative Real-Time Polymerase Chain Reaction

<table>
<thead>
<tr>
<th></th>
<th>WT-saline</th>
<th>WT-MK</th>
<th>ApoE−/−-saline</th>
<th>ApoE−/−-MK</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dL)</td>
<td>137.0±4.8</td>
<td>136.6±11.2</td>
<td>984.0±53.7**</td>
<td>1043.6±87.4**</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>21.5±1.5</td>
<td>21.2±2.0</td>
<td>100.3±17.2**</td>
<td>93.6±13.4**</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>10.5±1.0</td>
<td>10.7±1.0</td>
<td>300.3±22.2**</td>
<td>335.5±18.1**</td>
</tr>
<tr>
<td>MK (ng/mL)</td>
<td>0.274±0.044</td>
<td>0.515±0.044*</td>
<td>0.391±0.049</td>
<td>0.774±0.12**†</td>
</tr>
</tbody>
</table>

*P<0.05 and **P<0.01 vs. WT-saline. 1P<0.01 vs. WT-MK. *P<0.01 vs. ApoE−/−-saline. n=15 for ApoE−/−-saline and ApoE−/−-MK; n=10 for WT-saline and WT-MK. WT, wild-type; ApoE−/−, apolipoprotein E-knockout; TC, total cholesterol; TG, triglyceride; LDL, low-density lipoprotein; MD, midkine.
mined by assessing lipid deposition (Figure 2A). Compared with ApoE−/−-saline mice, ApoE−/−-MK mice had a significantly larger percent area of atherosclerotic lesions (26.3±0.6% vs. 6.8±1.8%, Figure 2B). In ApoE+/+ mice, no significant difference in the percent area of atherosclerotic lesions was observed between WT-saline and WT-MK mice. Specifically, aortas from the four different mouse groups were divided into 4 sections (arch, upper, middle, and lower aorta), and each section was analyzed separately (Figure 2C). In ApoE−/− mice, MK substantially increased the atherosclerotic lesions at the lower aortic sections, including the common iliac artery. There was no significant difference in atherosclerotic areas between WT-saline, WT-MK, and ApoE−/−-saline mice, indicating that under atherogenic conditions, MK specifically accelerates the progression of atherosclerosis in the iliac bifurcation. Although atherosclerotic lesions at the aortic arch were significantly increased in ApoE−/−-saline mice than in WT-saline and WT-MK mice, MK significantly increased the atherosclerotic lesions in ApoE−/− mice. The cholesterol levels in ApoE−/− mice were significantly higher than those in ApoE+/+ mice, as previously reported,30,31 whereas MK administration did not induce lipid profile changes in either ApoE+/+ or ApoE−/− mice (Table A).

**MK Increases the mRNA Levels of Pro-Inflammatory and Angiogenic Factors**

The mRNA levels of different factors were examined in extracts from the entire aorta. In ApoE−/− mice, MK administration was associated with significantly higher levels of pro-inflammatory factors (interleukin 1α [IL1A], interleukin 1β [IL1B], chemokine ligand 2 [CCL-2], interferon γ [IFNG], basic fibroblast growth factor [bFGF], and hepatocyte growth factor [HGF]) in entire aorta extracts is shown for the 4 groups (n=5 for each group) at 20 weeks of age using quantitative real-time polymerase chain reaction (PCR) analysis. *P<0.01 vs. WT-saline, †P<0.01 vs. WT-MK, ‡P<0.01 vs. ApoE−/−-saline mice. Abbreviations as in Figure 1.
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foam cells detected using the Mac-3 antibody showed significantly larger Mac-3-positive areas within the lesions of aortic roots from ApoE−/−-MK mice, compared with the corresponding areas in ApoE−/−-saline mice (Figure 4A). Statistical analysis revealed that these differences were statistically significant (Figure 4B), suggesting that the increase in the atherosclerotic areas as a result of MK administration is most likely caused by an increase in the number of macrophages localized in these lesions. In addition to the effect of MK on macrophages, we also investigated the

Macrophages and VSMCs Migrate Into Atherosclerotic Lesions

Representative images of macrophages and macrophage-

Figure 4. MK enhances macrophage migration into the atherosclerotic lesions. (A) Paraffin-embedded sections of aortic roots from 20-week-old ApoE−/−-saline and ApoE−/−-MK mice were stained with antibodies against Mac-3 (brown) and counterstained with hematoxylin (blue). Representative staining patterns for ApoE−/−-saline and ApoE−/−-MK mice are shown. (B) Percentage of Mac-3-positive areas relative to the total area of the aortic root (ApoE−/−-saline and ApoE−/−-MK mice, n=5 each). **P<0.01 vs. ApoE−/−-saline mice. Abbreviations as in Figure 1.

Figure 5. MK decreases the number of apoptotic cells in atherosclerotic lesions in ApoE−/− mice. (A) Apoptosis was quantified in ApoE−/−-saline and ApoE−/−-MK mice using a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling staining (TUNEL) assay. Co-staining with DAPI (blue) was performed to identify nuclei. The upper panel shows representative DAPI-stained nuclei (blue), the middle panel shows TUNEL-positive cells (green), and the bottom panel shows a merged image. (B) The TUNEL-positive nuclei/total nuclei percentage was used to quantify the apoptotic cells. The data presented are the means of 3 independent areas in the aortic valve region from 5 different ApoE−/−-saline and ApoE−/−-MK mice. *P<0.05 vs. ApoE−/−-saline mice (n=5 each). Abbreviations as in Figure 1.

kin 1β [IL1B], C-C motif chemokine ligand 2 [CCL2], and interferon γ [IFNG]) and angiogenic factors (basic fibroblast growth factor [bFGF], and hepatocyte growth factor [HGF]), as shown in Figure 3. Such MK treatment-induced pro-inflammatory and angiogenic effects were not observed in wild-type mice.
In ApoE +/+ mice, MK tended to decrease the mRNA levels of α-smooth-muscle actin-positive lesion areas in atherosclerotic areas within the lesions in aortic roots. The mRNA levels of α-smooth-muscle actin-positive lesion areas tended to be more abundant in ApoE−/−-MK mice than in ApoE−/−-saline mice, but the difference was not statistically significant (12.7±1.2% vs. 10.4±1.3%, P=0.19). MK significantly increased the mRNA levels of bFGF and HGF in inflammatory cells, supporting the hypothesis that IFNG promotes atherosclerosis.

MK Preferentially Inhibits Macrophage Apoptosis In Vivo

Apoptotic cells were detected by TUNEL staining (Figure 5A). The ratio of the TUNEL-positive area relative to the DAPI-positive area was significantly smaller in ApoE−/−-MK lesions than in ApoE−/−-saline lesions (0.9±0.2% in ApoE−/−-MK mice vs. 2.6±0.8% in ApoE−/−-saline mice; P=0.01). The majority of the TUNEL-positive areas were located in the central atheroma, rather than in the smooth muscle cell-rich lesion cap, implying that MK administration preferentially inhibited macrophage apoptosis. The mRNA levels of Bcl-2 and Bax, members of the Bcl-2 family of cell death regulatory proteins, extracted from the entire aorta were evaluated by using real-time PCR. In ApoE−/− mice, MK treatment increased the mRNA levels of Bcl-2, whereas it decreased the mRNA levels of Bax, resulting in a decrease in the Bax/Bcl-2 ratio (Figure 6). In ApoE−/− mice, MK tended to decrease the mRNA levels of Bax, leading to a decrease in the Bax/Bcl-2 ratio, but there was no significant effect of MK on the Bax/Bcl-2 ratio in wild-type mice.

Discussion

In the present study, we assessed the effects of long-term systemic administration of MK on atherosclerosis in ApoE−/− and wild-type mice. Whether MK enhances or inhibits the progression of atherosclerosis in atherosclerotic models without mechanical injury has been uncertain because MK has contradictory effects: it enhances VSMC migration and proliferation in addition to inhibiting macrophage apoptosis (promoting atherosclerosis), whereas it repairs EC dysfunction (inhibiting atherosclerosis). Importantly, its recovery effects on ECs are critical for the early stage of atherosclerosis, because EC deterioration in spontaneously developed lesions can greatly influence the subsequent progression of atherosclerosis. This is the first study, to our knowledge, that investigated the effects of continuous MK administration in ApoE−/− and wild-type mice. We found that under atherogenic conditions, MK administration enhanced early-stage atherosclerosis in an ApoE−/− mouse model without mechanical injury, by increasing pro-inflammatory and angiogenic factors. MK also enhanced the migration of macrophages into atherosclerotic lesions and inhibited apoptosis in those lesions, leading to plaque formation.

Pro-Inflammatory Factors (IL1A, IL1B, IFNG) and Angiogenic Factors (bFGF, HGF)

Experimental data from mouse atherosclerosis models have shown that IL1A and IL1B have pro-atherogenic properties associated with the upregulation of endothelial adhesion molecules and the activation of macrophages and vascular cells. Moreover, postnatal inhibition of IFNG prevents the progression of existing atherosclerotic plaques and stabilizes lipid and macrophage-rich advanced plaques, suggesting that IFNG has athero-progressive effects. This result indicates that MK may have paracrine and/or direct effects on immunoreactive cells resulting in the production of IFNG.

Local administration of bFGF in the adventitia induces the development of vasa vasorum and accelerates plaque progression in ApoE−/− mice, supporting the notion that vasa vasorum formation plays a crucial role in the pathogenesis of atherosclerosis. HGF induces EC migration and proliferation, and angiogenesis. In the present study, MK significantly increased the mRNA levels of bFGF and HGF in inflammatory cells, supporting the hypothesis that MK enhances atherosclerosis in ApoE−/− mice. Interestingly, the MK-induced significant increase in the mRNA levels of pro-inflammatory (IL1A, IL1B and IFNG) and angiogenic (bFGF and HGF) factors was observed only in ApoE−/− mice and not in wild-type mice. Thus, the role of MK under atherogenic conditions might be different from that under normal conditions, and further investigations are required to show the underlying mechanism.

Macrophage and VSMC Migration

Macrophage migration into atherosclerotic plaques is critical for the pathogenesis of atherosclerosis. In vitro studies using a Boyden chamber assay have demonstrated that MK enhances macrophage migration. In the present in vivo study, immunohistochemistry analysis showed that MK enhanced macrophage migration in the atherosclerotic lesions (Figure 4). In the in vitro study by Horiba et al., MK had a direct effect on macrophage migration. However, in in vivo studies, many factors can influence macrophage migration into atherosclerotic lesions, and cross-talk between macrophages and other cell types can also occur. For example, ECs release the chemokines monocyte chemotactic protein-1 (MCP-1) and macrophage colony-stimulating factor (M-CSF), which recruit...
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Figure 7. Schematic summarization illustrating the possible effects of midkine (MK) on the process of atherosclerotic plaque formation. The solid lines illustrate the known pathways that give rise to lesion cells. The most important points of this study are: (1) MK promotes macrophage migration into atherosclerotic lesions; (2) MK enhances vascular smooth muscle cell (VSMC) migration into the sub-endothelial space and produces extracellular matrix, forming the tunica intima of the artery; and (3) MK inhibits macrophage and foam cell apoptosis.

Monocytes into atherosclerotic lesions and differentiate monocytes into macrophages. Taken together, the results of these previous reports and our present study results that found that the mRNA level of CCL2 (MCP-1) in entire aorta extracts was elevated, and this suggests that MK might have a direct effect on ECs, causing the release of CCL2.

VSMC migration is also important for the formation of atherosclerotic lesions. According to a well-known theory, VSMCs migrate into the sub-endothelial space in response to injury or inflammatory stimuli, and produce extracellular matrix, forming the tunica intima of the artery. Following their migration to the intima, VSMCs gradually resume a quiescent phenotype. The effect of MK on the enhancement of VSMC migration in vitro has been published. Taken together, these results suggest that in ApoE−/− mice, MK activates macrophage and VSMC migration, leading to the progression of atherosclerosis (Figure 7).

Anti-Apoptotic Effect of MK
Apoptotic cell death occurs during the development and progression of atherosclerotic plaques. Among the various cell types that undergo apoptosis in atherosclerotic lesions, macrophages are the predominant type. VSMC apoptosis can destabilize the fibrous cap and induce rupture. In such lesions, adequate efferocytosis is sufficient to protect against atherosclerosis. Apoptotic cells and the phagocytes that engulf them produce anti-inflammatory cytokines, such as transforming growth factor-β and interleukin-10, which inhibit the early stages of atherosclerosis development. In the present study, we found that the number of TUNEL-positive cells within the plaques was significantly lower in the MK-treatment group, and the apoptotic cells were estimated to be macrophages. In terms of mRNA levels, the ratio of the pro-apoptotic factor, Bax, to the anti-apoptotic factor, Bcl-2, was significantly lower in ApoE−/−-MK mice than in ApoE−/−-saline mice (Figure 6), suggesting that MK has an anti-apoptotic effect on atherosclerotic lesions under atherogenic conditions. These anti-apoptotic effects of MK are in agreement with recently reported findings in a congestive heart failure mouse model.

Regarding clinical manifestations of atherogenesis, serum MK levels are significantly increased in obese human subjects, and MK is present in the thickened intima of fatty streaks and advanced atherosclerotic regions in human atheromatous plaques. Moreover, serum MK levels were significantly higher in patients with severe peripheral artery disease than in healthy controls. A recent impressive clinical study demonstrated the use of MK as a diagnostic tool for coronary atherosclerosis in humans, yet basic research investigating the direct effect of MK on atherosclerosis is missing. To the best of our knowledge, this is the first study demonstrating that long-term MK administration promotes atherosclerotic plaque formation in ApoE−/− mice. Our data suggest that higher serum MK levels may play a role in atherogenesis, especially under atherogenic conditions, such as hyperlipidemia and/or obesity. Taken together, our results suggest that maintaining or reducing serum MK levels under atherogenic conditions would inhibit atherosclerotic plaque formation; therefore, MK inhibition could be used in upstream therapies targeting atherosclerosis. Further studies are needed to validate this hypothesis.

Future Aspects
The present study showed that MK had an athero-progressive effect in ApoE−/− mice, indicating that MK might be a molecular target for the treatment of atherosclerosis. Further investigations are required to confirm the indications for
the use of MK inhibitors (antisense oligoDNA, siRNA, and RNA aptamer) to prevent atherosclerosis progression.

### Study Limitations

First, the mouse model used in the present study can be regarded as representative of an intermediate and not an early stage of atherosclerosis (the mice were fed a high-fat diet for 12 weeks). Second, and related to apoptosis, the TUNEL staining used in the present study is not specific for apoptosis. We did not directly show the co-existence of macrophages and apoptotic cells in the aortic lesions, and we did not identify the types of apoptotic cells. Third, we did not investigate the effect of MK on EC dysfunction in the present study. Moreover, we did not confirm the direct effect of MK by a genetic modification approach, such as double knockout of MK and ApoE. We did not investigate MK expression in atherosclerotic lesions in ApoE−/− mice. Moreover, we did not investigate the time-course of blood MK levels in ApoE−/− mice.

### Conclusions

In conclusion, we found that the systemic administration of MK in apolipoprotein-E knockout mice increased atherosclerosis through its pro-inflammatory, angiogenic, and anti-apoptotic functions, suggesting that MK may be a therapeutic target for the prevention of atherosclerosis under atherogenic conditions.

### Conflicts of Interest

The authors declare that they do not have anything to disclose regarding funding or conflicts of interest with respect to this manuscript.

### Author Contributions

Y.T. designed the animal study, performed the animal surgeries, completed the experiments, analyzed the data, performed the statistical analyses, and wrote the manuscript. M. Horiba designed and supervised the project, interpreted the results, and wrote the manuscript. M. Harada designed and supervised the project. K.S. performed experiments and analyzed the data. K.T. and T.M. provided suggestions on the project and revised the manuscript. K. Kadomatsu and K. Kamiya supervised the project.

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