Lack of Mitogen-Activated Protein Kinase Phosphatase-1 Protects ApoE-Null Mice Against Atherosclerosis


Rationale: Multiple protein kinases have been implicated in cardiovascular disease; however, little is known about the role of their counterparts: the protein phosphatases.

Objective: To test the hypothesis that mitogen-activated protein kinase phosphatase (MKP)-1 is actively involved in atherogenesis.

Methods and Results: Mice with homozygous deficiency in MKP-1 (MKP-1−/−) were bred with apolipoprotein E-deficient mice (ApoE−/−) and the 3 MKP-1 genotypes (MKP-1+/+; MKP-1−/−/ApoE−/− and MKP-1−/−/ApoE−/−) were maintained on a normal chow diet for 16 weeks. The 3 groups of mice exhibited similar body weight and serum lipid profiles; however, both MKP-1−/− and MKP-1−/− mice had significantly less aortic root atherosclerotic lesion formation than MKP-1+/+ mice. Less en face lesion was observed in 8-month-old MKP-1−/− mice. The reduction in atherosclerosis was accompanied by decreased plasma levels of interleukin-1α and tumor necrosis factor α, and preceded by increased antiinflammatory cytokine interleukin-10. In addition, MKP-1−/− mice had higher levels of plasma stromal cell–derived factor-1a, which negatively correlated with atherosclerotic lesion size. Immuno-histochemical analysis revealed that MKP-1 expression was enriched in macrophage-rich areas versus smooth muscle cell regions of the atheroma. Furthermore, macrophages isolated from MKP-1−/− mice showed dramatic defects in their spreading/migration and impairment in extracellular signal-regulated kinase, but not c-Jun N-terminal kinase and p38, pathway activation. In line with this, MKP-1−/− atheroma exhibited less macrophage content. Finally, transplantation of MKP-1−/− macrophages into MKP-1−/− mice fully rescued the wild-type atherosclerotic phenotype.

Conclusion: These findings demonstrate that chronic deficiency of MKP-1 leads to decreased atherosclerosis via mechanisms involving impaired macrophage migration and defective extracellular signal-regulated kinase signaling. (Circ Res. 2010;106:00-00.)

Key Words: mitogen-activated protein kinases ■ protein phosphatases ■ atherosclerosis ■ macrophage ■ cell migration

Atherosclerosis is a chronic inflammatory disease involving complex interactions among multiple modified lipoproteins, monocyte-derived macrophages, T lymphocytes, endothelial cells, and smooth muscle cells. It is generally believed that endothelial dysfunction is one of the key initiating steps in the pathogenesis of atherosclerosis. Specifically, activation of vascular endothelial cells by various stimuli, including oxidatively modified lipoproteins and inflammatory cytokines, increases the expression of adhesion molecules such as E-selectin, vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 on the endothelial cell surface, leading to increased rolling, adhesion and transmigration of monocytes into the vascular wall. Infiltrated monocytes then differentiate into macrophages, which produce more inflammatory mediators and become foam cells after uptake of oxidized low-density lipoprotein (LDL) via scavenger receptors SR-A and CD36.

The above scenario of atherogenesis requires cell signaling mechanisms involving multiple protein kinases, including the mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. Although much information has been obtained regarding the roles of various protein kinases in the pathogenesis of atherosclerosis, little is known about the role of their counterparts: protein phosphatases. Among the ≈147 human protein phosphatases, none have been rigorously demonstrated to play a role in atherogenesis, although many have been implicated in cancer. A recent study showed...
that the mitogen-activated protein kinase phosphatase (MKP)-1 is required for oxidized LDL-induced monocyte adhesion to vascular endothelial cells. In line with this observation, MKP-1 has been shown to be highly expressed in the atherosclerotic lesions of mouse aorta. These findings suggest a potential role of MKP-1 in atherogenesis.

MKP-1 belongs to a family of dual-specificity protein phosphatases that differ in their substrate specificity, tissue distribution, inducibility by extracellular stimuli and cellular localization. An established function of MKP-1 is inactivating MAPKs by causing dephosphorylation of ERK, JNK, and p38 at specific tyrosine and threonine residues. MKP-1 is an immediate early gene and its encoding protein is primarily localized to the nucleus. It is upregulated by many factors, including oxidative stress, heat shock, lipopolysaccharide (LPS), and some peptide ligands, such as angiotensin and tumor necrosis factor (TNF)alpha.

Atherosclerotic Lesion Analysis

Because MKP-1-null mice have been reported to be resistant to high-fat diet-induced obesity, and to exclude the confounding factors of obesity on atherosclerosis formation, we fed mice with normal chow diet and then evaluated atherosclerosis lesion size at aortic sinus and en face entire aorta. The mouse heart and aorta were perfused, dissected, and subjected to quantification of atherosclerosis as previously described.

PCR Genotyping

Primer sets: MKP-1 forward-1: 5'-CCAGGTACTCTGTCGTTGGTGC-3'; MKP-1 forward-2: 5'-TGCTGCTTCTTTACTGAAGGC-3'; MKP-1 reverse: 5'-CCGGACATCTCTCTATGAC-3'; ApoE forward-1: 5'-GGCTAGCCAGGGAGAGCC-G-3', ApoE forward-2: 5'-TGGACTTGAGGAAGGCTCGAGC-3', and ApoE reverse: 5'-GCCGCCC-CGACTGATCT-3'.

Lipid Analysis and Lipoprotein Profile Measurement

Mouse plasma was fractionated by protein liquid chromatography. Cholesterol in the column eluate was combined with Infinity cholesterol reagent (Thermo Electron, Melbourne, Australia) as previously described. Areas under the cholesterol elution curve were integrated and identified as very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), LDL, or high-density lipoprotein (HDL) based on their coelution with human lipoproteins. Plasma total cholesterol was measured with the same reagent following the manufacturers' instructions.

Mouse Cytokine/Chemokine Array Assay

Mouse plasma levels of 40 cytokines/chemokines were screened and determined using the “Mouse Cytokine Array Panel A Array Kit” (R&D Systems, Minneapolis, Minn), according to the user manual.

Luminex Bead-Based Multiplexing Assay

A customized “Mouse Cytokine 6-Plex” kit (LINCOplex, Millipore) was used according to the user manual to quantify interleukin (IL)-1alpha, IL-1beta, IL-10, interferon-inducible protein-10, macrophage inflammatory protein-1, and TNFa levels in mouse plasma.

Enzyme-Linked Immunosorbent Assay

Mouse plasma SDF-1alpha and IL-10 concentrations were determined using mouse SDF-1alpha and IL-10 Quantikine ELISA kits (R&D Systems) according to the respective user manuals.

Immunohistochemical Analysis

Mouse hearts were sectioned, fixed and processed for antibody staining. The following antibodies were used: anti-MKP-1 (V-15, Santa Cruz Biotechnology, Santa Cruz, Calif; 1:50 dilution); anti-Mac-3 (BD Biosciences; 1:500 dilution); and anti-alpha-smooth muscle actin (Sigma; 1:500 dilution).
Macrophage Infiltration Assay

Peritoneal macrophages from MKP-1−/− ApoE−/− mice and MKP-1+/− ApoE−/− mice were harvested with 5 mL PBS 3 days after the intraperitoneal injection of thioglycollate. Cells that had infiltrated the peritoneal area in response to thioglycollate were counted.

Boyden Chamber Cell Migration Assay

Cell migration was performed with a modified Boyden chamber system as previously described.27 Peritoneal macrophages added to the upper chambers of the Boyden chambers were attracted overnight by 10% FBS in the lower chamber, after which the number of cells that migrated to the underside of the membrane were counted.

Cell Adhesion and Spreading Assay

Peritoneal macrophages were seeded into six-well plates. After seeding for 2 hours, the number of macrophages attached to the plates was counted and compared between MKP-1−/− ApoE−/− mice and MKP-1+/− ApoE−/− mice. The cell morphology (spreading) difference between wild-type and MKP-1-null macrophages was observed after 2 days in culture.

Western Blot Analysis

Peritoneal macrophages were seeded in 6-well plates for 48 hours (10% FBS in DMEM), after which the cells were serum-starved (0.1% FBS in DMEM) for 4 hours. The cells were then treated with 10% FBS for different time points. Cells were then lysed and standard Western blotting was performed as previously described.27

Bone Marrow Transplantation

At 6 weeks of age, ApoE-null and MKP-1/ApoE-double null female mice were lethally irradiated (9 Gy) using a cesium gamma source. Four hours later, 1×10⁷ bone marrow cells from the donor mice were injected into the tail veins of the recipient mice. The bone marrow-transplanted mice received normal chow diet for an additional 16 weeks. At the end of 22 weeks, the mice were euthanized and atherosclerotic lesions in the aortic sinus were determined as described above. Animal procedures were performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Data Analysis

Data are expressed as means±SEM. Means of 2 groups were compared using Student t test (unpaired, 2-tailed) and one-way ANOVA was used for comparison of more than 2 groups with P<0.05 considered to be statistically significant.

Results

Metabolic Characteristics of MKP-1+/+ ApoE−/−, MKP-1+/− ApoE−/−, and MKP-1−/− ApoE−/− Mice

Because MKP-1–null mice are known to be resistant to high-fat diet–induced obesity,23 we fed mice normal chow diet to avoid potential confounding factors from differences in body weights. As shown in Figure 1, mice of the 3 genotypes fed with normal chow diet for 16 weeks showed no difference in body weights for males or females (Figure 1A and 1B). In addition, the heart weights among the 3 groups of mice also were not statistically different in males or females (Figure 1C and 1D). Serum total cholesterol as well as HDL, LDL, VLDL, and IDL levels were nearly identical among the 3 groups of the male or female mice.

Figure 1. Effect of MKP-1 deficiency on metabolic characteristics of ApoE-null mice. Body (A and B) and heart (C and D) weights in male and female mice fed a normal chow diet for 16 weeks. Serum level of total cholesterol and its different components were determined as described in Methods for male (E) and female (F) mice fed a normal diet for 16 weeks. Numbers in columns indicate the number of mice in that group.
mice (Figure 1E and 1F). No difference was observed in cholesterol distributions of HDL, LDL, VLDL, and IDL among the 3 groups of male or female mice (data not shown).

### Analysis of Atherosclerosis in MKP-1+/+ApoE−/−, MKP-1+/−ApoE−/−, and MKP-1−/−ApoE−/− Mice

After 16 weeks of normal chow diet, aortic sinus and en face assays were performed to evaluate lesion formation in MKP-1–deficient mice. We observed limited atherosclerotic lesion formation using an en face staining approach in the aortic arch, thoracic and abdominal aorta after 16 weeks of normal diet; this was not affected by MKP-1 deficiency (Online Figure I). However, atherosclerotic lesion size in the aortic sinus was markedly reduced in both the MKP-1+/+ApoE−/− and MKP-1+/−ApoE−/− mice as compared to MKP-1+/+ApoE−/− mice (∼50% reduction), but only in the female mice (Figure 2B and 2C). To test whether this gender difference was attributable to a low level of lesion formation in the male ApoE-null control mice, we extended the feeding time to 32 weeks for the male mice. MKP-1–intact male mice at 32 weeks (Figure 2D) exhibited almost 10-fold greater lesion size than at 16 weeks (Figure 2B) in their aortic sinus. In this set of experiments, compared to controls, we observed an ∼50% reduction in lesion size in the MKP-1+/−ApoE−/− mice, but not in the MKP-1+/+ApoE−/− mice (Figure 2D). Further analysis of the entire aorta from 8-month-old male mice showed a significant reduction (∼60%) of en face lesion area in MKP-1+/−ApoE−/− mice compared with MKP-1+/+ApoE−/− mice (Figure 2E).

### Plasma Levels of Cytokines/Chemokines in MKP-1+/+ApoE−/−, MKP-1+/−ApoE−/−, and MKP-1−/−ApoE−/− Mice

To determine whether the reduced atherosclerotic lesion is associated with less inflammation, we screened 40 cytokines/chemokines in mouse plasma using a microarray approach. We found that 20 of the 40 plasma cytokines/chemokines were downregulated in MKP-1−/−ApoE−/− mice as compared to MKP-1+/+ApoE−/− (Figure 3A and Online Figure II). In MKP-1+/−ApoE−/− mice, many cytokines/chemokines were also downregulated (35 of the 40) (Figure 3A and Online Figure II). Notably, there was only one chemokine, SDF-1α, that was expressed at a much higher level in both MKP-1+/−ApoE−/− mice and MKP-1−/−ApoE−/− mice than in the control MKP-1+/+ApoE−/− mice (Figure 3A and Online Figure II). To confirm this observation, we used an ELISA assay to quantify the plasma levels of SDF-1. We found that the plasma SDF-1 level was nearly 3-fold higher in MKP-1+/−ApoE−/− and MKP-1−/−ApoE−/− mice than in the control mice in the female but not male groups (Figure 3B and 3C). Analysis of 32 plasma samples from the 3 groups of mice at 16 weeks revealed that the plasma level of SDF-1 was negatively correlated with atherosclerotic lesion size (Figure 3D). Luminex bead–based multiplexing assay showed that the absence of MKP-1 decreased plasma levels of IL-1α and TNFα (Figure 3E and 3F), but not interferon-inducible protein-10, macrophage inflammatory protein-1α and IL-1β (Online Figure III). Interestingly, MKP-1 deficiency did not change the level of antiinflammatory cytokine IL-10 at 4 months, but IL-10 levels were significantly higher at 2 and 3 months in MKP-1–null mice as compared to MKP-1–intact mice (Figure 3G).
MKP-1 Expression in Atherosclerotic Lesions

To determine the localization of MKP-1 protein in the mouse aortic sinus, we performed an Immuno-histochemical study. We found that MKP-1 protein expression was enriched in the atheroma of 16-week-old ApoE-null mice (Figure 4), whereas virtually no staining was observed in MKP-1–null mice (Online Figure IV), indicating the specificity of the MKP-1 antibody. Figure 4 also shows that MKP-1 protein expression was highly concentrated in the macrophage-rich (Mac-3 staining), versus smooth muscle–rich (SMC actin staining) regions, suggesting a potential link between macrophage function and MKP-1-deficiency-mediated attenuation of atherosclerosis.

Macrophage Function of MKP-1–/- ApoE–/- Mice Versus MKP-1+/+ApoE–/- Mice

To assess possible functional defects in macrophages from MKP-1–null mice, we focused on cell adhesion and migration because we have recently shown that MKP-1 deficiency leads to decreased vascular endothelial cell migration.17 Figure 5A shows that macrophages isolated from MKP-1+/+ ApoE–/- mice exhibited much less spreading capacity than macrophages from MKP-1+/+ApoE–/- mice. However, macrophage adhesion at 2 hours was not different between these 2 genotypes (data not shown). Because cell spreading is highly related to cell migration, we further examined macrophage migration in vitro and in vivo. Figure 5C shows that peritoneal injection of thioglycollate for 3 days elicited macrophage infiltration to the peritoneum with mean cell number $3.3 \times 10^6$/mL in MKP-1+/+ApoE–/- mice, which was reduced to $1.8 \times 10^6$/mL in MKP-1–/-ApoE–/- mice. To further confirm a defect in macrophage migration in MKP-1–/-ApoE–/- mice, we isolated peritoneal macrophages and seeded them into the Boyden chamber system to test macrophage migration in vitro. Figure 5B shows that the number of MKP-1–null macrophages migrating toward serum was significantly less than the number of MKP-1–intact macrophages. In line with these observations, we further found that MKP-1 deficiency led to decreased
macrophage content in the atheroma as evidenced by reduced Mac-3 staining (Figure 6).

**Activation of MAPK Pathways in Macrophages of MKP-1+/ApoE−−− Mice Versus MKP-1+/ApoE−−− Mice**

We compared the time courses of MAPK pathway activation, including ERK, JNK and p38, in macrophages with or without MKP-1 in the ApoE-null background. Figure 7 shows that macrophages isolated from MKP-1+/ApoE−−− and ApoE−−−− mice exhibited no serum stimulation of the ERK pathway, whereas ERK was transiently activated by serum as expected in macrophages from MKP-1+/− and ApoE−−−− mice. No significant difference in the kinetics of either JNK or p38 activation by serum between MKP-1+/− and MKP-1−−−− macrophages was observed (Figure 7A). To assess the connection between defective ERK signaling and impaired macrophage migration, peritoneal macrophages were pretreated with U0126, a selective MEK1/2 inhibitor, and then stimulated with serum. Figure 7B shows that the ERK pathway inhibitor decreased serum-induced migration of MKP-1−−−− cells, with a negligible effect on MKP-1−−−− cells, suggesting that defective ERK signaling may be one of the mechanisms responsible for impaired cell migration.

**Figure 6.** Macrophage content in atheroma of MKP-1−−−− mice. Macrophage spreading capacity was observed by cell morphology after 2 days in culture. B. Macrophage migration in vitro in response to 10% FBS was determined using a Boyden chamber system. Left, Representative images of Boyden chamber membranes containing migrated cells. C. The number of thioglycollate-elicited macrophages in the peritoneum was counted and compared between the indicated groups of mice. Numbers in columns indicate the number of mice in that group. *P<0.05 vs respective controls.

**Figure 7.** MAPKs activation in macrophage of MKP-1−−−− mice with ApoE−−−− background. The time courses of ERK, JNK, and p38 phosphorylation in response to serum were determined by Western blotting in macrophages of female mice fed normal chow diet for 16 weeks. Data are expressed as fold increases over control after normalization to the respective controls. A. The experiment was repeated 3 times (3 mice in each genotype) with similar results. B. The effect of U0126 on migration capacity of MKP-1−−−− vs MKP-1−−−− macrophages was determined using a Boyden chamber system. *P<0.05 vs respective controls.
Figure 8. Contribution of MKP-1 in bone marrow–derived cells to atherosclerotic lesion formation. Female MKP-1−/−/ApoE−/− and MKP-1+/−/ApoE−/− mice were lethally irradiated at 6 weeks and transplanted with donor bone marrow (MKP-1+/−/ApoE−/−) as indicated. Mice were continuously fed with normal chow diet for additional 16 weeks after bone marrow transplantation, after which they were euthanized for evaluation of atherosclerotic lesion size in the aortic sinus. Age-matched (22 weeks) female mice served as nontransplanted controls.

Analysis of Atherosclerotic Lesions in Mice Having Undergone Bone Marrow Transplantation

Because a recent study showed a potential role of endothelial MKP-1 in controlling adhesion molecule expression,28 bone marrow transplantation was used to determine the contribution of MKP-1 in blood cells to the development of atherosclerosis. Figure 8 shows that in nontransplanted age-matched (22 weeks) control female mice, the lack of the MKP-1 gene led to a ∼50% reduction in aortic sinus atherosclerotic lesion formation as expected. The lesser atherosclerotic lesion phenotype in the MKP-1−/− mice was fully restored to the level of MKP-1−/− intact mice by transplanting MKP-1−/− intact bone marrow into the MKP-1−/− mice at 6 weeks (maintained for additional 16 weeks, Figure 8). These results suggest that macrophage MKP-1 may be more important than endothelial MKP-1 in optimal initiation and progression of atherosclerosis.

Discussion

In the present study, we show for the first time that genetic deletion of the MKP-1 gene reduced atherosclerosis in ApoE-null mice fed with normal chow diet. We also found that MKP-1 deficiency led to decreased plasma levels of proinflammatory cytokines IL-1α and TNFα, preceded by increased antiinflammatory cytokine IL-10. In addition, we unexpectedly observed that the absence of MKP-1 yielded a “normal” plasma level of SDF-1 in ApoE-null mice, and that SDF-1 levels negatively correlated with atherosclerotic lesion size. Furthermore, we demonstrated that MKP-1 deficiency prevented macrophage spreading in culture and attenuated migration in vitro and in vivo.

Finally, we found a macrophage signaling defect in the ERK MAPK pathway in the absence of MKP-1. MKP-1 is highly expressed in atheroma and is required for monocyte adhesion to cultured endothelial cells activated by oxidized LDL.7 However, it has not been determined whether lesional MKP-1 is a potential therapeutic target, ie, is increased MKP-1 part of a compensatory mechanism limiting lesion development or is it fundamentally proatherogenic? The results of the present study reveal that loss of MKP-1 results in a favorable outcome in an animal model of atherosclerosis. Thus, in the absence of MKP-1, mice on the ApoE-null background on a normal chow diet for 16 weeks (32 weeks for male mice) had a 50% reduction in lesion size compared with ApoE-null mice with intact MKP-1. This is in spite of similarly elevated levels of total cholesterol and comparable levels of LDL and HDL in the 2 groups of mice. It is also striking to see that even deletion of one copy of the MKP-1 gene led to a similar degree of reduction in atherosclerosis compared with MKP-1−/− null homozygous mice, at least in females, suggesting that MKP-1 may be a vital molecule for maximal progression of atherosclerosis. On the other hand, we found that MKP-1−/− intact ApoE-null mice fed with normal chow diet for 16 weeks exhibited limited aortic en face lesion (<1.0%), which was not statistically different from the MKP-1−/− null mice. Though this result differs from our observations in the aortic sinus and we are aware that not all sites of the aorta show the same degree of lesion development,29 it should be noted that normal chow diet for 16 weeks is of limited value for assessing a potential antiatherogenic effect on the entire aorta because of the low number and small size of lesions in the control group.

Multiple risk factors are implicated in the pathogenesis of atherosclerosis, including metabolic abnormalities, such as hyperlipidemia, obesity and diabetes.1,2 In this study, we did not find significant difference in the lipid profile between MKP-1−/− null and MKP-1−/− intact mice in the ApoE-deficient background, suggesting that reduced atherosclerosis caused by MKP-1 deficiency is not attributable to a change in lipid metabolism. This is further supported by the fact that MKP-1 deletion did not change the body and heart weights in the ApoE-null mice. It should be noted, however, that mice lacking MKP-1 are resistant to high-fat diet–induced obesity.23 This apparent discrepancy could be explained by multiple differences in experimental conditions, including normal chow diet versus high fat diet, young versus old mice, and ApoE-null versus ApoE-intact background.

It is well established that atherosclerosis is a chronic inflammatory disease.1,2 In line with this concept, our present study showed that loss of MKP-1 led to decreases of multiple proinflammatory cytokines in MKP-1−/− null mice, including TNFα and IL-1α, whereas the antiinflammatory cytokine IL-10 was increased at 2 and 3 months, but not in the fourth month. These results suggest that MKP-1 deletion leads to upregulation of antiinflammatory cytokine during atherosclerosis initiation phase, and may explain in part decreased expression of proinflammatory cytokine. These data are closely associated and consistent with the reduced atherosclerosis results. However, this result does differ from other studies. For example, several studies showed that MKP-1 deletion render mice more vulnerable to LPS-induced endotoxic shock and death, which was accompanied by upregulation of proinflammatory cytokines.
such as TNFα and IL-1β in serum. The exact mechanism(s) responsible for this apparent discrepancy remains to be determined, however, a key difference between the present study and the prior studies is the use of mice lacking ApoE. Total serum cholesterol was ~4-fold higher in these mice versus wild-type mice, and this pathological level of cholesterol and its derived oxidized lipids may be considered chronic inflammatory stimuli on the mice. In addition, we measured the cytokine/chemokine level at 16 weeks, whereas prior studies tested within hours or <2 days after LPS injection. Thus, it is conceivable that whether MKP-1 deficiency leads to a proinflammatory or an antiinflammatory phenotype may depend on the experimental model used, particularly acute versus chronic models. Indeed, Chi et al have shown that LPS challenge of the MKP-1–null mice first led to an acute increase of the proinflammatory cytokine TNFα, but later, TNFα decreased and the antiinflammatory cytokine IL-10 became dominant. We believe this interpretation may also explain the disparity between the present study and a recent study in an acute inflammation model by Zakkar et al, who showed that endothelial MKP-1 suppresses proinflammatory gene activation at sites in the aorta that are resistant to atherosclerosis. The finding by Zakkar et al would suggest the possibility that aortic atherosclerosis in the MKP-1–null mice could be increased because of increased adhesion molecule expression. It should be noted that Zakkar et al used ApoE-intact mice without assessment of atherosclerotic lesions, and they determined endothelial adhesion molecule expression within just several hours of LPS injection. Although we did not measure endothelial adhesion molecule expression in our animal model, our study showed that transplantation of MKP-1–intact bone marrow into MKP-1–null mice fully rescued the wild-type atherosclerotic phenotype, suggesting that the macrophage MKP-1 may be more important than the endothelial MKP-1 in optimal formation of atherosclerotic lesions.

Another intriguing finding of this study is that MKP-1 deficiency appears to maintain a normal plasma level of SDF-1α in female ApoE-null mice, and more importantly, the plasma level of SDF-1α was negatively correlated with the lesion size of atherosclerosis. These results are consistent with a report showing that in healthy men, the average plasma level of SDF-1α is ~3.5 ng/mL, whereas it is <1.0 ng/mL in patients with unstable angina pectoris, a complication of advanced atherosclerotic lesion/plaque rupture. Although the exact role of SDF-1α in atherosclerosis is not well understood and beyond the scope of the present study, our finding, together with the reports of others, point to a role for SDF-1α in the pathogenesis of atherosclerosis. Furthermore, plasma SDF-1α levels may represent a new prognostic factor for atherosclerosis. SDF-1α is primarily expressed in pancreas and liver, with low expression in vascular smooth muscle and endothelial cells. Thus, it is reasonable to see a relatively high level of circulating SDF-1α in blood in physiological condition. We propose that circulating SDF-1α may be atheroprotective by keeping leukocytes inside the bloodstream, preventing them from transmigrating into the tissues/ organs. In addition, SDF-1α may also have some protective effects on endothelium.

It is well known that early stage fatty lesions are predominantly composed of lipid-enriched macrophages differentiated from infiltrated monocytes. Our results showed that MKP-1 was mainly expressed in the macrophage-rich, rather than smooth muscle-rich, areas of the aortic sinus. This observation led us to propose that macrophage functions may be altered in the MKP-1–null mice. Evidence supporting this hypothesis include: (1) macrophages isolated from MKP-1–null mice showed a defect in their spreading capacity when compared to macrophages from control mice; (2) macrophage infiltration into the peritoneum in response to thioglycollate was decreased ~50% in MKP-1–null mice compared with MKP-1–intact mice; (3) our Boyden chamber cell migration study showed that the number of MKP-1–null macrophages migrating toward serum was significantly less than the number of MKP-1–intact macrophages; (4) we found that the ERK MAPK signaling pathway was impaired in MKP-1–null macrophages; and (5) importantly, we confirmed that macrophage content was much lower than that in the controls, and that ERK pathway inhibition decreased the migration capacity of MKP-1–intact cells to the level of MKP-1–null cells. These results strongly suggest that our observed reduction in atherosclerotic lesions in MKP-1–null mice is, at least in part, attributable to defective spreading and migration of macrophages. Of note, a similar defective migration capacity was observed in MKP-1–null vascular endothelial cells and more recently reported in MKP-1–null lymphocytes. In addition, our finding that transplantation of MKP-1–intact bone marrow into the MKP-1–null mice fully rescued the wild-type atherosclerotic phenotype provided another line of strong evidence in support of our contention. The molecular basis of defective ERK signaling in MKP-1–null mice needs to be explored in the future. It is plausible that deletion of MKP-1 in a chronic inflammation condition, such as ApoE-null background, may compensatorily upregulate other phosphatase(s) that selectively interfere with the ERK pathway. Also, we cannot exclude the possibility of an involvement of histone H3, which recently was identified as a new substrate for MKP-1.

In summary, we report the first evidence that in ApoE-null mice fed normal chow diet, the absence of MKP-1 leads to reduced atherosclerotic lesion formation, which is accompanied by a decreased expression of multiple cytokines, including TNFα and IL-1α. In addition, MKP-1 deficiency maintains a normal plasma level of SDF-1α, which is negatively associated with the degree of atherosclerosis. Furthermore, our results suggest that defective macrophage migration and ERK signaling underlie MKP-1-deficiency-mediated reduction in atherosclerosis. These findings highlight a new view that protein phosphatases may be as important as protein kinases in the pathogenesis of atherosclerosis.

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Disclosures

None.

Novelty and Significance

Although many studies show involvement of protein kinases in the formation of atherosclerosis, less is known about protein phosphatases. Using mice deficient in both mitogen-activated protein kinase phosphatase (MKP-1) and apolipoprotein (ApoE), we found that: (1) genetic deletion of the prototype phosphatase MKP-1 decreases aortic atherosclerosis; (2) contrary to previous work, lack of MKP-1 in this chronic inflammation model (ApoE-null background) result in the development of an antiinflammatory phenotype; (3) MKP-1 deficiency impairs macrophage migration and infiltration; and interestingly; (4) extracellular signal-regulated kinase 1/2 signaling pathway is attenuated in MKP-1–null macrophages. These findings demonstrate that MKP-1 is a positive regulator of inflammation in vivo and cell signaling pathways that are causally involved in the etiology of atherosclerosis. These findings demonstrate the importance of regulating protein phosphatases in atherogenesis and defined MKP-1 as a potential novel drug target for atherosclerosis therapy.

What Is Known?

- Several protein kinases have been shown to be involved in experimental atherogenesis, but the roles of protein phosphatases are less well known.
- MKP-1 has been shown as a negative regulator of MAPK signaling and acute inflammation.
- MKP-1 deletion leads to impaired endothelial cell migration.

What New Information Does This Article Contribute?

- This study shows for the first time that the prototype phosphatase, MKP-1, is actively involved in atherogenesis through a macrophage-based mechanism.
- In a model of chronic inflammation, MKP-1 acts as a positive regulator of MAPK signaling and inflammation.
- MKP-1 deficiency impairs macrophage adhesion and migration.

References


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Animal Procedures. Mice homozygous for inactivation of MKP-1 were back-crossed more than 10 generations into the C57BL/6 background. These MKP-1−/− mice were intercrossed with the ApoE-deficient mice also in the C57BL/6 background (Jackson Laboratory) to generate mice heterozygous at both loci. These MKP-1+/− ApoE−/− mice were back-crossed with ApoE−/− mice to produce MKP-1+/− ApoE−/− mice. Subsequently, the MKP-1+/− ApoE−/− offspring were bred to obtain the mice with the following three genotypes: MKP-1+/+ ApoE−/−, MKP-1+/− ApoE−/−, and MKP-1−/− ApoE−/−. At 4-week, male and female littermates were separated and divided into the three genotypes. Mice were fed standard chow diet with 4.5% fat (PMI Feeds, St. Louis, MO). All mice were maintained in a facility free of well-defined pathogens under the supervision of the Biological Resource Unit at Cleveland Clinic Lerner Research Institute. All animal protocols were approved by the Cleveland Clinic Institutional Animal Care and Use Committee.

Atherosclerotic Lesion Analysis. Because MKP-1−null mice have been reported to be resistant to high fat diet-induced obesity, and to exclude the confounding factors of obesity on atherosclerosis formation, we fed mice with normal chow diet for 16 weeks or 8 months and then evaluated atherosclerosis lesion size in aortic sinus and en face of entire aorta by Oil Red O staining as reported elsewhere. The mouse heart and aorta were perfused, dissected, and subjected to quantification of atherosclerosis, as previously described. Briefly, to quantify atherosclerosis in the aortic sinus, the heart was embedded in optimal cutting temperature (OCT) medium and frozen, after which serial sections (10 μm) were taken from the aortic sinus. Images were obtained of the sections following staining with Oil Red O (counterstaining with hematoxylin) and lesion area was quantified using Image-Pro software (Media Cybernetics). The mean atherosclerotic lesion size expressed as μm² for each animal was determined by averaging 4-6 sections from each mouse, using 80 μm intervals between the sections. For en face analysis, the stained aortas were opened longitudinal and fixed on agarose gel pads for photographing through a binocular microscope. Total and stained areas were quantified by Photoshop 7.0 software. Consistent with reports of others and our previous study, ApoE-null mice fed normal chow diet for 16 weeks did not develop significant atherosclerotic lesions in the arch, thoracic aorta or abdominal aorta (less than 1%). However, significant en face lesion in the entire aorta was detectable at 8-month time point (~7% of total en face area).

Mouse Genotyping by Polymerase Chain Reaction (PCR). Genomic DNA was extracted from mouse tail biopsy using the DNeasy Kit (Qiagen). A triple-primer method was used for genotyping apoE, MKP-1 and their mutants with neomycin-resistant gene inserts, respectively, using the following primer sequences: MKP-1 forward-1: 5’-CCAGGTACTGTGTCGGTGGTGC-
MKP-1 forward-2: 5’-TGCCTGCTCTTTACTGAAGGCTC-3’, MKP-1 reverse: 5’-CCTGGCACAATCCTCCTAGAC-3’; ApoE forward-1: 5’-GCCTAGCCGAGGGAGAGCCG-3’, ApoE forward-2: 5’-TGTGACTTGGGAGCTCTG-CAGC-3’, and ApoE reverse: 5’-GCCGCCC-CGACTGCATCT-3’. The PCR condition was as follows: jump start for 2 min at 95°C, denaturation for 1 min at 95°C, annealing for 1 min at 56°C, and extension at 72°C for 1 min, for 40 cycles. The resulting PCR products were resolved on a 1.5% agarose ethidium bromide gel and the amplified bands were visualized with ultraviolet light, after which the PCR products were purified and sequence identity was confirmed by sequencing.

**Lipid Analysis and Lipoprotein Profile Measurement.** Mice were fasted for 4 h before blood samples were collected by heart puncture. Plasma was separated by centrifugation and stored at -80°C. Plasma (5µL) was fractionated by fast protein liquid chromatography on tandem Superose 6 HR columns (GE Healthcare, Piscataway, NJ). Cholesterol in the column eluate was continuously monitored on-line by combining the eluate with Infinity cholesterol reagent (Thermo Electron, Melbourne, Australia) essentially as previously described. Area under the cholesterol elution curve was integrated and regions were identified as VLDL, IDL, LDL or HDL based on their co-elution with isolated human lipoproteins. Plasma total cholesterol was measured with the same reagent following the manufacturers’ instructions.

**Mouse Cytokine/Chemokine Array Assay.** Mouse plasma levels of 40 cytokines and chemokines were screened and evaluated using the “Mouse Cytokine Array Panel A Array Kit” (R&D Systems, Minneapolis), according to the user manual. The signals were detected using the ECL kit (PerkinElmer Life Science, Inc.), exposed on BioMax film (Kodak) and digitized using a CANON scanner. The image files were analyzed using Quantity One software (Bio-Rad). Data were expressed as pixel density.

**Luminex Bead-based Multiplexing Assay.** A customized “Mouse Cytokine 6-Plex” kit (LINCOplex, MILLIPORE) was used at 25°C according to the user manual to quantitate interleukin-1α (IL-1α), IL-1β, IL-10, IP-10, MIP-1α, and TNFα levels in mouse plasma. Serial dilutions of the lyophilized standard were prepared in assay diluent and transferred to appropriate microtiter wells containing diluted antibody-coated bead complexes and incubation buffer. Each plasma sample (25 µL) was transferred to appropriate wells containing diluted antibody-coated bead complexes and incubation buffer. Samples were incubated for 16 h at 4°C. After washing with assay wash buffer (200 µL/well), detection antibody cocktail (25 µL) was added into each well and incubated for 1 h at room temperature. After washing, streptavidin-phycoerythrin (25 µL) was added to each well and incubated for 30 min. After a final wash and added sheath fluid (100 µL), the plate was analyzed using the Luminex 100 analyzer (Luminex...
At least 400 events (beads) were collected for each cytokine/sample and median fluorescence intensities were obtained. Cytokine concentrations were calculated based on standard curves using Graphpad Prism version 4 (Graphpad Software, San Diego, CA).

**ELISA Assay.** Mouse plasma SDF-1α concentrations were determined using a mouse SDF-1 Quantikine ELISA kit (R&D Systems, Minneapolis) according to the user manual. Plasma levels of IL-10 were also determined from 1- to 4-month old mice using a mouse IL-10 Quantikine ELISA kit (R&D Systems, Minneapolis) according the user manual.

**Immuno-histochemical Analysis.** After perfusion, the mouse heart (16-week old female mice) was isolated and snap-frozen fixed and embedded in OCT. The samples were sectioned, fixed in acetone, and processed for antibody staining according to standard protocols. The following antibodies were used: anti-MKP-1 (V-15, Santa Cruz Biotechnology, Inc., 1:50 dilution); anti-Mac-3 (BD Biosciences, 1:500 dilution); anti-a-smooth muscle actin (Sigma, 1:500 dilution); and a universal detecting antibody kit VECTASTAIN ABC (Vector Laboratories, Inc, Burlingame, CA). Sections were counterstained with Hematoxylin. For macrophage quantification, at least 5 sections per animal were analyzed for each staining. Digital images were acquired and analyzed using Image-Pro software (Media Cybernetics). For each stain, the threshold was predetermined, and held constant for all sections analyzed from each protocol. The relative content of macrophages was expressed as Mac-3-positive area as a percent of per plaque area, and the data were collected in a blinded manner by one observer.

**Macrophage Infiltration Assay.** Peritoneal macrophages from MKP-1⁻/⁻ApoE⁻/⁻ mice and MKP-1⁺/⁺ApoE⁻/⁻ mice fed regular chow diet were harvested with 5 mL PBS 3 days after the intra-peritoneal injection of thioglycollate. Cells that had infiltrated into the peritoneal area in response to thioglycollate were counted in a blinded manner.

**Boyden Chamber Cell Migration Assay.** Cell migration was performed with a modified Boyden chamber trans-well system (BD Biosciences), as previous described. Briefly, pre-coated cell culture inserts having 4 µm pore size membranes were placed into 24-well plates. Peritoneal macrophages were isolated as described above and re-suspended in DMEM medium containing 0.5% FBS. Cell suspensions (150 µL, 10,000 cells) were added to the upper chambers (the inserts) of the Boyden chambers. The lower chamber was filled with 500 µL DMEM medium containing 10% FBS. Cells in the upper chamber were attracted overnight by 10% FBS in the lower chamber, after which the cells remaining on the upper surface of the membrane were removed by cotton swab. Inserts were then washed three times with PBS and cells on the underside of the membrane were fixed with cold methanol for 15 min and stained with Accustain (hematoxylin solution, Sigma) for 1 h. The membranes were washed again in
PBS, removed and mounted on glass slides. Cells were counted in a blinded manner. The assays were performed in triplicate wells for each genotyped mouse, and the experiment was repeated at least 3 times.

**Cell Adhesion and Spreading Assay.** Peritoneal macrophages were isolated as described above and re-suspended in DMEM medium containing 10% FBS. $10^5$ cells were seeded into each well of six well plates pre-coated with fibronectin. After seeding for 2 h, floating cells were washed away. The number of macrophages attached to the plates was counted in a blinded manner and was compared between MKP-1^-/-ApoE^-/- mice and MKP-1^-/-ApoE^-/- mice. The cell morphology (spreading) difference between wild type and MKP-1-null macrophages was observed after 2 d in culture and pictures were taken using a phase contrast microscope with a digital imaging system.

**Western Blot Analysis of Protein Phosphorylation.** Isolated peritoneal macrophages were seeded in 6-well plates for 48 h (10% FBS in DMEM), after which the cells were serum-starved (0.1% FBS in DMEM) for 4 h. The quiescent cells were then activated by 10% FBS for different time points. After stimulation, cells were lysed and a standard western blotting was performed as previously described. The individual primary antibodies used were anti-p-ERK1/2, anti-p-JNK, and anti-p-p38 (1:1000 dilutions, Cell Signaling). Equal protein loading was verified by stripping off the original antibodies, and the membranes were re-probed with the primary antibody anti-GAPDH (1:1000, Santa Cruz), or anti-total ERK, or p38 (1:1000, Cell Signaling).

**Bone Marrow Transplantation.** At 6 weeks of age, ApoE-null and MKP-1/ApoE-double null female mice were lethally irradiated (9 Gy) from a cesium gamma source. Four hours later, $1\times10^7$ bone marrow cells from the 6-week old female donor mice were injected into the tail veins of the recipient mice. The bone marrow-transplanted mice received normal chow diet for additional 16 weeks. At the end of 22 weeks, the mice were euthanized and atherosclerotic lesions in the aortic sinus were determined as described above. Animal procedures were performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

**Data Analysis.** Data are expressed as means ± S.E.M. Means of two groups were compared using Student’s t-test (unpaired, two-tailed) and one-way ANOVA was used for comparison of more than two groups with $p<0.05$ considered to be statistically significant.

**REFERENCES**


Online Fig. I. Plaque formation in the aorta of MKP-1-intact versus MKP-1-null mice. ApoE-null female mice were fed with normal chow diet for 16 weeks, after which en face lesion of entire aorta including aortic arch, thoracic and abdominal aorta was detected by Oil red O staining.

Online Fig. I. Plaque formation in the aorta of MKP-1-intact versus MKP-1-null mice. ApoE-null female mice were fed with normal chow diet for 16 weeks, after which en face lesion of entire aorta including aortic arch, thoracic and abdominal aorta was detected by Oil red O staining.
Online Fig. II. Effect of MKP-1 deficiency on mouse plasma levels of 40 cytokines/chemokines. Plasma samples pooled from 3 mice with each of the MKP-1 genotype in the ApoE-null background were screened by a micro-array approach detailed in “Materials and Methods”. Data were expressed as pixel density and relative change of concentrations for the individual cytokines/chemokines was considered significant if the pixel density showed more than 15% difference among the three groups of mice.
Online Fig III. Lacking MKP-1 does not change plasma level of IP-10, MIP-1α & IL-1β in female ApoE-null mice. Plasma levels of IP-10 (A), MIP-1α (B) and IL-1β (C) at 16-week were determined by a luminex bead-based multiplexing assay detailed in “Materials and Methods”. Numbers in columns indicate the No. of mice in that group.
Online Fig. IV. MKP-1 protein expression is enriched to atheroma of ApoE-null mice. Immuno-histochemical staining of MKP-1 protein in aortic sinus sections of representative 16-week-old ApoE-null mice. Brown color stands for MKP-1 positive staining and blue color was from Hematoxylin staining. The numbers shown in right side refer to microscopy amplification fold.
Online Fig. V. No effect of MKP-1 deficiency on cell differentials of peritoneal leukocytes. Peritoneal leukocytes were isolated 72 hours after peritoneal stimulation with thioglycollate and cell differentials were determined morphologically by a senior medical technologist after Wright staining in slides. Three random fields were chosen for cell counting in each slide and each genotype had 3 slides from 3 mice. Total counted cell number is 9000 in each genotype. Cell type, other than macrophages, lymphocytes and neutrophils was not observed.
Online Fig. VI. Effect of MKP-1 deficiency on cytokine/chemokine mRNA expression in peritoneal macrophages. Peritoneal macrophages were isolated 72 hours after peritoneal stimulation with thioglycollate, after which total RNA were isolated for cDNA synthesis. Real-time PCR was performed to compare the relative mRNA expression levels of the indicated genes in MKP-1-intact vs. MKP-1-null cells. Data were from 3 mice (female, 16-week) for each genotype and were normalized by GAPDH. MKP-1 wild type was set as “control”. *, p<0.05 to respective controls.
Online Fig. VII. No difference of plasma SDF-1a level between MKP-1 wild type and knockout mice with intact ApoE background. Plasma level of SDF-1a was determined by ELISA assay in 16-week-old ApoE-intact female mice fed with normal chow diet. Numbers in columns indicate the No. of mice in that group.