Inhibition of Bone Morphogenetic Proteins Protects Against Atherosclerosis and Vascular Calcification

Yucheng Yao,* Brian J. Bennett,* Xuping Wang, Michael E. Rosenfeld, Cecilia Giachelli, Aldons J. Lusis, Kristina I. Boström

Rationale: The bone morphogenetic proteins (BMPs), a family of morphogens, have been implicated as mediators of calcification and inflammation in the vascular wall.

Objective: To investigate the effect of altered expression of matrix Gla protein (MGP), an inhibitor of BMP, on vascular disease.

Methods and Results: We used MGP transgenic or MGP-deficient mice bred to apolipoprotein E mice, a model of atherosclerosis. MGP overexpression reduced vascular BMP activity, atherosclerotic lesion size, intimal and medial calcification, and inflammation. It also reduced expression of the activin-like kinase receptor 1 and the vascular endothelial growth factor, part of a BMP-activated pathway that regulates angiogenesis and may enhance lesion formation and calcification. Conversely, MGP deficiency increased BMP activity, which may explain the diffuse calcification of vascular medial cells in MGP deficient aortas and the increase in expression of activin-like kinase receptor 1 and vascular endothelial growth factor. Unexpectedly, atherosclerotic lesion formation was decreased in MGP-deficient mice, which may be explained by a dramatic reduction in expression of endothelial adhesion molecules limiting monocyte infiltration of the artery wall.

Conclusions: Our results indicate that BMP signaling is a key regulator of vascular disease, requiring careful control to maintain normal vascular homeostasis. (Circ Res. 2010;107:485-494.)

Key Words: bone morphogenetic protein □ matrix Gla protein □ atherosclerosis □ vascular calcification □ inflammation

Inflammation and atypical cell differentiation are hallmarks of atherosclerotic lesion development and are regulated by systemic and local factors. Bone morphogenetic proteins (BMP)-2 and -4, multifunctional growth factors and morphogens, have been implicated as mediators of endothelial inflammation in response to proatherogenic oscillatory shear stress, oxidative stress and proinflammatory cytokines.1,2 Several BMPs have also been detected in calcified atherosclerotic plaques,3,4 and BMP-2 promotes calcification of aortic myofibroblasts,5 consistent with the ability of BMPs to induce bone differentiation.6 Thus, the morphogenetic activities of BMP-2 and -4 might directly influence lesion progression through endothelial inflammation and cell differentiation.

Gene deletion in mice previously identified matrix Gla protein (MGP) as an essential inhibitor of arterial calcification,7 which prevents osteochondrogenic lineage reprogramming of smooth muscle cells (SMCs).8 Excessive MGP expression, on the other hand, has no discernable effect on the aortic vascular wall, but leads to growth inhibition of the pulmonary vascular tree through interference with BMP-4 signaling.9 MGP antagonizes BMP signaling10,11 through direct protein-protein interaction involving essential proline and γ-carboxylated glutamate residues in MGP.12 It acts as a negative feedback regulator in a BMP-activated pathway that is triggered by interaction between BMP-2 or -4 and the activin-like kinase receptor (ALK)2, first stimulating expression of the activin-like kinase receptor (ALK)1, and then expression of the vascular endothelial growth factor (VEGF) and MGP itself.9,11,13 This regulatory pathway is likely to play an important role in embryonic development because ALK1 and VEGF are essential for angiogenesis,13,14 BMP and MGP affects pattern formation in vascular mesenchymal cells,15 and MGP promotes SMC differentiation.8 Here, we hypothesize that perturbations in BMP-signaling play important roles in the development of vascular disease.

Methods

Animals and Diets
ApoE−/− mice on a C57BL/6J background were purchased from The Jackson Laboratory (Bar Harbor, Me). MGP+/− mice, generated in...
our laboratory on a C57BL/6J background,9 were crossed with Apoe−/− mice to generate MGPtg/wt;Apoe−/− mice. We used the MGPtg/wt mice because the phenotype was apparent in MGP−/− mice, and a low birth rate of MGP−/− mice made it difficult to obtain MGP−/− mice.9 MGP−/− mice on C57BL/6J background2 were obtained from Dr Gerard Karsenty (Columbia University, New York) and crossed with the Apoe−/− mice to generate MGP−/−; Apoe−/− mice. All mice were maintained on standard chow (Diet 8604, Harlan Teklad, Laboratory, Madison, Wis). At 8 to 10 weeks of age, littermate Apoe−/− and MGPtg/wt; Apoe−/− mice were either continued on standard chow or switched to a high-fat/high-cholesterol diet (Western diet) (Research Diets, New Brunswick, NJ, diet #D12108, containing 21% fat [wt/wt], 1.25% cholesterol [wt/wt]) for 16 weeks. The studies were reviewed by the Institutional Review Board and conducted in accordance with the animal care guidelines set by the University of California, Los Angeles. See the expanded Methods section, available in the Online Data Supplement at http://circres.ahajournals.org, for details regarding animals, lipid and phosphate analyses, lesion and calcification quantification, validation of antibodies, immunoblotting, RNA analysis, immunohistochemistry and immunofluorescence, ELISA, complete blood count, and statistical analysis.

Results

Reduced Aortic Atherosclerotic Lesion Formation in MGPtg/wt; Apoe−/− Mice

To explore the ability of BMP signaling to influence vascular disease, we took advantage of the availability of transgenic mice expressing human MGP (MGPtg/wt) and mice with MGP gene deletion (MGP−/−).7,8 These were crossed with Apolipoprotein E null (Apoe−/−) mice, a common model of atherosclerosis and lesion calcification.

We first determined the effect of increased MGP expression on atherosclerotic lesions and arterial calcification by comparing Apoe−/− and MGPtg/wt; Apoe−/− mice after 16 weeks on Western diet. The hypercholesterolemia of the Apoe−/− and MGPtg/wt; Apoe−/− mice was exacerbated by the Western diet, but there were no statistically significant differences in body mass or lipid levels in male or female mice, respectively (Online Table I). Cross-sectional analysis of the atherosclerotic lesions showed statistically significant differences between the two strains. The Apoe−/− mice had an average aortic sinus lesion area of 540,117 μm² and 589,159 μm² in males (n=15) and females (n=11), respectively, whereas MGPtg/wt; Apoe−/− mice had an average area of 338,312 μm² and 216,890 μm² in males (n=12, P=0.0023) and females (n=7, P=0.0013), respectively (Figure 1A and 1B). The lower number of females was attributable to difficulties in obtaining sufficient females carrying the MGP transgene.9 En face analysis of whole aortas showed similar differences between the two strains. The Apoe−/− mice had an average aortic lesion area of 8.5% and 9.1% in males (n=5) and females (n=4), respectively, whereas MGPtg/wt; Apoe−/− mice had an area of 4.2% and 4.1% in males (n=6, P=0.038) and females (n=4, P=0.018), respectively (Figure 1C and 1D). Thus, enhanced MGP expression limited formation of atherosclerotic lesions.

Reduced Aortic Atherosclerotic Lesion Calcification in MGPtg/wt; Apoe−/− Mice

Because of the well-defined role of MGP as an inhibitor of vascular calcification, we examined the effect of enhanced MGP expression on calcification. We first compared total calcium in aortic tissue in Apoe−/− and MGPtg/wt; Apoe−/− mice after 16 weeks of regular chow diet (CH) or Western diet (high fat diet, HF). The results showed no significant effect of the Western diet in the respective strain (Figure 2A). However, total calcium was significantly decreased in both chow and fat-fed MGPtg/wt; Apoe−/− mice compared to the similarly fed Apoe−/− mice (Figure 2A). We next compared lesion calcification by cross-sectional analysis (schematically illustrated in Figure 2B) in the Apoe−/− and MGPtg/wt; Apoe−/− mice that were fed a Western diet. This showed that both the rate and amount of lesion calcification per animal were significantly lower in MGPtg/wt; Apoe−/− mice than in Apoe−/− mice. The rate of calcification was 61.5% and 31.6% in the Apoe−/− and the MGPtg/wt; Apoe−/− mice, respectively (Figure 2C), and the average amount of calcification was 16.7 μm² (n=13) in Apoe−/− mice but only 1.2 μm² in MGPtg/wt; Apoe−/− (n=6) (Figure 2D and 2E). Only animals that exhibited calcification were analyzed, and males and females were analyzed together because of the low calculation rate in the MGPtg/wt; Apoe−/− mice. Thus, enhanced MGP expression limited total aortic calcium and lesion calcification. Similar to previous reports,16 we observed that the Apoe−/− mice developed cartilage metaplasia and medial calcification in parts of the media without lesions. We found that 61.5% of the Apoe−/− mice, but none of the MGPtg/wt; Apoe−/− mice, had medial calcification (Figure 2F), suggesting that MGP also limits medial calcification. Because of the important role of phosphate in stimulating vascular calcification, we compared serum phosphate levels in the different mice, but there were no statistically significant differences (Online Table II).

Diminished Vascular BMP Activity in MGPtg/wt; Apoe−/− Mice

To examine BMP signaling in atherosclerotic vessels and its response to enhanced MGP expression, we first compared
expression of BMP-2, -4, and MGP in Apoe−/− and MGP<sup>tg/WT</sup>, Apoe−/− mice after 16 weeks of chow or Western diet. Aortic expression of BMP-4 increased 3-fold in both groups of mice on the Western diet compared to chow, whereas expression of BMP-2 remained the same (Figure 3A). Glyceraldehyde-3-phosphate dehydrogenase was used as reference gene for the PCR reactions. BMP-4 protein in serum and aorta also increased, as determined by ELISA and immunoblotting with quantification by densitometry (Figure 3B and 3C). β-Actin was used as loading control. Aortic expression of MGP was 3-fold higher in chow-fed MGP<sup>tg/WT</sup>;Apoe−/−/− mice than in Apoe−/−/− mice. It increased 3-fold in the Apoe−/− mice and 15-fold in the MGP<sup>tg/WT</sup>;Apoe−/−/− mice on the Western diet (Figure 3A), suggesting a synergistic effect of the transgene and the Western diet.

We next examined MGP in the vascular wall by immunohistochemistry. In aortic segments without obvious atherosclerotic lesions, MGP was detected mostly in proximity to the endothelium and the adventitia in chow-fed Apoe−/−/− mice (Figure 3D). Because Western diet and the transgene increased MGP expression, the staining in these areas intensified and MGP staining also appeared in the media (Figure 3D). Control experiments using MGP<sup>−/−</sup> aorta and the same MGP-antibodies showed no staining (Figure 3E). In atherosclerotic lesions, MGP and BMP-4 were detected in the same areas (Figure 3F), but the MGP expression was higher relative to BMP-4 in the MGP<sup>tg/WT</sup>;Apoe−/−/− mice than in the Apoe−/− mice (Figure 3F).

We predicted that BMP signaling activity would be less in the aortic walls of the MGP<sup>tg/WT</sup>;Apoe−/−/− mice because of the enhanced MGP expression. To test this, we determined the levels of activated, phosphorylated (p)SMAD1/5/8 in aortas from Apoe−/− and MGP<sup>tg/WT</sup>;Apoe−/−/− mice fed chow or a Western diet. The level of pSMAD1/5/8 was lower in the MGP<sup>tg/WT</sup>;Apoe−/−/− aortas than in the Apoe−/− aortas as determined by immunoblotting with densitometry (Figure 3G) and immunohistochemistry (Online Figure II). Total SMAD and pSMAD2/3, which mediate TGF-β signaling, were included for comparison and showed no change. Furthermore, pSMAD1/5/8 levels were diminished in atherosclerotic lesions from MGP<sup>tg/WT</sup>;Apoe−/−/− mice compared to Apoe−/−/− mice as determined by immunofluorescence (Figure 3H). Thus, the enhanced MGP expression in MGP<sup>tg/WT</sup>;Apoe−/−/− mice diminished BMP signaling.

**Increased MGP Expression Suppresses Expression of ALK1 and VEGF**

Expression of ALK1 is stimulated by BMP, an effect that is mediated by the ALK2 receptor. ALK1 is essential for normal angiogenesis, and has a dual role in VEGF regulation. ALK1 suppresses VEGF expression when stimulated by BMP-9, a circulating BMP that has been referred to as a vascular “quiescence” factor. Yet, ALK1 also enhances VEGF levels by inducing the ALK5 receptor, which reacts with TGF-β1 to increase VEGF expression. To examine this pathway, we determined aortic expression of ALK2, ALK1 and VEGF. The aortic expression of ALK1 and VEGF was increased in Apoe−/− mice on Western diet compared to chow, and suppressed in MGP<sup>tg/WT</sup>;Apoe−/−/− mice on both chow and Western diet as determined by real-time PCR.
Increased MGP Expression Suppresses Gene Expression Associated With Inflammation

Inflammation is a hallmark of atherosclerotic lesions and is partly mediated by BMP-2 and -4.1,2 To examine the effect of MGP overexpression on inflammatory activity, we compared aortic expression of CD68, a macrophage marker,20 interleukin (IL)-6,21 heat shock protein (HSP)70, a stress-induced protein,22 and the endothelial adhesion molecules intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin in Apoe−/− and MGPtg/wt;Apoe−/− mice. The results showed that aortic expression of all proteins increased with Western diet in the Apoe−/− mice, but not in the MGPtg/wt;Apoe−/− mice as determined by real-time PCR and immunoblotting with densitometry (Figure 5A through 5C). The real-time PCR results in the mice on Western diet were also normalized to lesion area, which did not significantly alter the results (Online Figure IV). The serum levels of IL-6 and HSP70 reflected the changes in the aortic expression (Figure 5D). Furthermore, expression of all proteins decreased in lesions of MGPtg/wt;Apoe−/− mice compared to Apoe−/− mice as determined by immunofluorescence (Figure 5E). Thus, the results suggested that inflammation is reduced by MGP overexpression.

Reduced Formation of Atherosclerotic Lesions in MGP−/−;Apoe−/− Mice

MGP−/− mice are known to develop arterial calcification,7,8 and our results showed increased aortic pSMAD1/5/8 levels (Online Figure V), suggesting increased BMP activity. As previously reported, the calcification also caused structural changes in the aortic wall (see H&E staining; Online Figure VA, top).7,8
To determine whether lesion formation occurred in MGP deficiency, we compared MGP−/−:Apoe−/− mice to Apoe−/− and MGP+/−:Apoe−/− mice. The MGP+/−:Apoe−/− mice were unable to tolerate a Western diet because of its severe phenotype, which was indistinguishable from that of MGP−/− mice. All mice were therefore maintained on a chow diet for 18 weeks. As expected, the MGP+/−:Apoe−/− mice were smaller than the Apoe−/− mice,7 but there were no significant differences in lipid levels (Online Table I). Cross-sectional analysis showed that the average aortic sinus lesion area was 99,981 μm² in chow-fed Apoe−/− mice (n=13), whereas lesion formation was minimal, 3,857 μm² in MGP−/−:Apoe−/− mice (n=5, $P=0.0231$) (Figure 6A and 6B). Males and the females were combined in the analysis because of the limited number of MGP−/−:Apoe−/− mice that survived 18 weeks. There were no significant differences between the Apoe−/− and the MGP+/−:Apoe−/− mice (Figure 6A). The MGP+/−:Apoe−/− mice demonstrated the same altered aortic wall structure as the MGP−/− mice, which prevented en face analysis.

Furthermore, total calcium in aortic tissue was highly increased in the MGP−/−:Apoe−/− mice (Figure 6C), and cross-sectional analysis of the aortas demonstrated the extensively calcified media in the MGP−/−:Apoe−/− mice (Figure 6D and 6E) compared to the Apoe−/− mice that had minimal calcification in both types of analysis, also when compared to Apoe−/− mice fed a Western diet (Figure 1). The rate of calcification was 100% in the MGP−/−:Apoe−/− mice compared to 0% in the Apoe−/− mice (Figure 6D). The average amount of calcification in the aorta was 73,136 μm³ (n=7, 30 μm aortic segments) in MGP−/−:Apoe−/− mice, and 0 μm³ in the Apoe−/− mice (n=13) (Figure 6C), consistent with previous reports.7,8 There were no significant differences between the Apoe−/− and the MGP+/−:MGP+/− mice. In addition, calcium formation without lesion formation was observed in the aortic valves of the MGP−/−:Apoe−/− mice, suggesting that the valvular BMP response was similar to that of the aortic wall. There were no significant differences in serum phosphate levels (Online Table II).

We then compared SMAD1/5/8 signaling and expression of ALK2, ALK1, VEGF, BMP-2 and -4 in the MGP−/−: Apoe−/− and the Apoe−/− mice. Again, the MGP-deficiency resulted in increased levels of pSMAD1/5/8, as determined by immunoblotting with densitometry and immunofluorescence (Figure 7A and 7B). Expression of ALK2, ALK1, and VEGF, but not BMP-2 and -4, was also enhanced in the MGP−/−: Apoe−/− mice as determined by real-time PCR, immunoblotting with densitometry and immunofluorescence (Figure 7C through 7E). Furthermore, expression of CV2, but not Noggin and Chordin, was significantly increased (Online Figure VI), supporting that CV2 is responsive to changes in BMP activity.
Diminished Inflammatory Response in MGP−/−;Apoe−/− Mice

To examine whether changes in the inflammatory response are involved in reducing lesion formation, we examined the expression of CD68, IL-6, HSP70 and endothelial adhesion molecules in the MGP−/−;Apoe−/− and the Apoe−/− mice. Expression of all proteins was dramatically reduced in the aortas of the MGP−/−;Apoe−/− mice compared to the Apoe−/− mice, as determined by real-time PCR, immunoblotting with densitometry and immunofluorescence (Figure 8A through 8D). No differences were detected in levels of circulating monocytes and other white blood cells as determined by standard methodology (Online Table III). The results suggest that reduced expression of endothelial adhesion molecules explains, at least in part, the decrease in lesion formation.

Discussion

BMP signaling in vertebrate development is essential for regulation of gastrulation, patterning, and organogenesis, whereas postnatal BMP signaling regulates injury repair, remodeling, and inflammation. In this study, we show that BMP signaling is a connecting link between hyperlipidemia, an inflammatory stimulus, and the atherogenic response in the arterial wall. We also present evidence that a BMP-activated regulatory pathway, which was identified in vitro and includes ALK2, ALK1, MGP, and VEGF, is active in vivo during the development of vascular disease.

The presence of the MGP transgene led to more efficient reduction in BMP activity and lesion formation in response to a high fat diet in Apoe−/− mice. It also led to less expression of ALK1 and VEGF, the precise role of which is likely dependent on location and timing. Suppression of ALK1 and VEGF in lesions may limit neoangiogenesis and reduce intraplaque hemorrhages and calcification, although a baseline expression of VEGF is required for maintenance and repair of a healthy endothelium. It is also known that VEGF is required for normal ossification during endochondral bone formation, and high VEGF levels might actually promote calcification. The beneficial effect of BMP inhibition, however, has to be weighed against the effect of limiting BMP in vascular growth considering that excess MGP inhibits angiogenesis and pulmonary vascular growth. It illustrates the delicate balance in BMP signaling that directs growth and homeostasis of different vascular beds.

We detected enhanced MGP expression close to the endothelium, in the media and in atherosclerotic lesions. It is consistent with previous reports of increased MGP expression in calcified media and atherosclerotic lesions. However, the presence of the MGP transgene led to more efficient reduction in BMP activity and lesion formation in response to a high fat diet in Apoe−/− mice. It also led to less expression of ALK1 and VEGF, the precise role of which is likely dependent on location and timing. Suppression of ALK1 and VEGF in lesions may limit neoangiogenesis and reduce intraplaque hemorrhages and calcification, although a baseline expression of VEGF is required for maintenance and repair of a healthy endothelium. It is also known that VEGF is required for normal ossification during endochondral bone formation, and high VEGF levels might actually promote calcification. The beneficial effect of BMP inhibition, however, has to be weighed against the effect of limiting BMP in vascular growth considering that excess MGP inhibits angiogenesis and pulmonary vascular growth. It illustrates the delicate balance in BMP signaling that directs growth and homeostasis of different vascular beds.

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involves an osteochondrogenic lineage reprogramming of SMCs, suggesting that such transdifferentiation of SMCs is driving calcification in the vascular wall. This effect appears to be dependent on locally expressed MGP given that only MGP expression targeted to SMCs, but not circulating MGP, is able to rescue the MGP\(^{-/-}\) mouse.\(^{32}\) Our results further suggest that it occurs in absence of inflammatory activation. A similar calcific response may be triggered by increased BMP signaling in the atherosclerotic lesions, supporting the general concept that developmental programs can be reactivated in vascular disease.

Only BMP-4 was found to increase in response to the high fat diet even though both BMP-2 and -4 have been implicated in proinflammatory effects in the endothelium and vascular calcification.\(^{1,2,3,33}\) Although we cannot exclude local variations of BMP-2 not detected in the analysis, it suggests that BMP-4 more readily initiates atherosclerotic development in mice. The expression of endothelial adhesion molecules and proteins associated with inflammation and cellular stress correlated well with increased BMP activity and lesion formation in the Apoe\(^{-/-}\) and MGP\(^{+/+}\), Apoe\(^{-/-}\) mice fed chow (CH) or Western (HF) diet, as determined by real-time PCR (A), immunoblotting with densitometry (B and C), and immunofluorescence (D). E, Serum levels of IL-6 and HSP70, as determined by immunoblotting with densitometry. **P<0.01, ***P<0.001 (Tukey’s test).

Interestingly, HDL, a well-known antiatherogenic factor, is able to induced expression of ALK2, which in turn allows induction of ALK1, VEGF, and MGP.\(^{17}\) Indeed, the Apo-AI transgenic mice, which have high HDL and minimal atherosclerosis, showed enhanced aortic expression of all these factors,\(^{17}\) which suggests that HDL protects against inflammation and atherosclerosis in part by influencing BMP signaling. However, it is not clear whether the protective effect derives from ALK2 activating several protective pathways or from early induction of ALK1, MGP, and VEGF before lesion formation.
In this study, we have focused on the role MGP as an inhibitor of BMP signaling. We have not evaluated other aspects of its role as a matrix protein, including potential changes in extracellular location and binding of calcium and other proteins in the extracellular matrix. For example, MGP may attach to vitronectin through its C terminus, which could alter its ability to bind BMP. The function of MGP is affected by factors that interfere with γ-carboxylation. For

Figure 6. Reduced lesion formation in extensively calcified arteries from MGP−/−; ApoE−/−, MGP+/−; ApoE−/−, and MGP−/−; ApoE−/− mice were maintained on chow for 18 weeks. Male and female mice were analyzed together. A, Mean lesion area. B, Representative oil red O-stained aortic sinus section from male and female ApoE−/− and MGP−/−; ApoE−/− mice. C, Total calcium in aortic tissues (n = 4). D, Calcification rate. E, Mean lesion calcification. ***P < 0.001 (Tukey’s test). In A and D, statistically significant differences were determined by the Mann-Whitney rank sum test.

Figure 7. Increased aortic BMP-signaling and expression of ALK1 and VEGF in MGP−/−; ApoE−/− mice. A and B, BMP activity in aortas of ApoE−/− and MGP−/−; ApoE−/− mice, as determined by immunoblotting with densitometry for pSMAD1/5/8 (A), and in lesions, as determined by immunofluorescence (B). Structural changes in aortas of MGP−/−; ApoE−/− mice are visible in the H&E-stained sections (B, top). C through E, Expression of ALK2, ALK1, VEGF, BMP-2, and BMP-4 in aortas from ApoE−/− and MGP−/−; ApoE−/− mice, as determined by real-time PCR (C) and immunoblotting with densitometry (D), and in lesions, as determined by immunofluorescence (E). **P < 0.01, ***P < 0.001 (Tukey’s test).
example, warfarin, a vitamin K antagonist, increases the levels of uncarboxylated, nonfunctioning MGP and may enhance calcification.\textsuperscript{12,35,36} It is also known that the reduction of vitamin K is less efficient in vascular SMCs than in the liver.\textsuperscript{35} Furthermore, MGP contains phosphorylated serines, which contribute to its function as a calcification inhibitor.\textsuperscript{37} However, it is not clear whether there is a relationship between the phosphorylation status of MGP and its effect on BMP signaling.

In summary, our results support the concept that modulation of BMP signaling through alterations in MGP expression regulates vascular disease, as well as the development of a normal arterial wall. A regulatory pathway triggered by BMP and involving ALK2, ALK1, VEGF, and MGP is central for vascular development, disease, and homeostasis. Understanding the role of vascular BMP may reveal new approaches to treat and prevent vascular disease.

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Disclosures
None.

References
Bone morphogenetic protein (BMP)-2 and -4 are multifunctional growth factors that mediate endothelial inflammation in response to proatherogenic stimuli in vitro.

BMP-2 and -4 have bone-inducing properties and are detected in calcified areas of atherosclerotic lesions.

Matrix Gla protein (MGP) binds and inhibits BMP-2 and -4. MGP deficiency leads to chondrogenic transdifferentiation and calcification of vascular smooth muscle cells (SMCs).

BMP-2 and -4 induce expression of the activin-like kinase receptor (ALK)1 is essential for angiogenesis and, in turn, regulates the vascular endothelial growth factor (VEGF).

Increased levels of MGP efficiently limit BMP activity, endothelial inflammation, atherosclerosis, and lesion calcification.

BMP activity regulates expression of ALK1 and VEGF in atherosclerotic vessels.

The vascular endothelium is abnormal in MGP deficiency; it lacks expression of endothelial adhesion molecules, which leads to less monocyte infiltration and atherosclerotic lesion formation.
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ONLINE SUPPLEMENT
Detailed Method Section

Animals and Diets

Apoe\textsuperscript{−/−} mice on a C57BL/6J background were purchased from Jackson Laboratory (Bar Harbor, ME). MGP\textsuperscript{tg/wt} mice, previously generated in our laboratory on a C57BL/6J background \textsuperscript{1} were crossed with Apoe\textsuperscript{−/−} mice to generate MGP\textsuperscript{tg/wt}\textsuperscript{;Apoe\textsuperscript{−/−}} mice. We used the MGP\textsuperscript{tg/wt} mice since the phenotype was apparent in MGP\textsuperscript{tg/tg} mice, and a low birth rate of MGP\textsuperscript{tg/tg} mice \textsuperscript{1} made it difficult to obtain MGP\textsuperscript{tg/tg} mice. MGP\textsuperscript{+/-} mice on C57BL/6J background \textsuperscript{2} were obtained from Dr. Gerard Karsenty (Columbia University, New York, NY) and crossed with the Apoe\textsuperscript{−/−} mice to generate MGP\textsuperscript{−/−;Apoe\textsuperscript{−/−}} mice. Genotypes were confirmed by PCR \textsuperscript{1, 3}, and experiments were performed with generation F4-F6. All mice were fed a standard chow diet (Diet 8604, Harlan Teklad, Laboratory, Madison, WI). At 8 to 10 weeks of age, littermate Apoe\textsuperscript{−/−} and MGP\textsuperscript{tg/wt}\textsuperscript{;Apoe\textsuperscript{−/−}} mice were either continued on standard chow or switched to a high-fat/high-cholesterol diet (Western diet) (Research Diets, New Brunswick, NJ, diet #D12108, containing 21% fat [w/w], 1.25% cholesterol [w/w]) for 16 weeks. Littermate MGP\textsuperscript{−/−;Apoe\textsuperscript{−/−}}, MGP\textsuperscript{+/-;Apoe\textsuperscript{−/−}}, MGP\textsuperscript{−/−}, and Apoe\textsuperscript{−/−} and wild type mice were maintained on standard chow. The studies were reviewed by the Institutional Review Board and conducted in accordance with the animal care guidelines set by the University of California, Los Angeles.

Lipid and Phosphate Analyses

Mice were fasted for 16 hours and anesthetized via exposure to isoflurane before blood was collected through the retroorbital sinus; plasma was stored at \(-80^\circ\text{C}\). Plasma triglyceride, cholesterol, high-density lipoprotein cholesterol (HDL-C), and free fatty acids were measured as previously described \textsuperscript{4}. Serum phosphate levels were measured using a QuantiChrom\textsuperscript{™} Phosphate Assay kit as per manufacturer’s instructions (BioAssay Systems, Hayward, CA).

Lesion Quantification

The mice were euthanized and perfusion fixed with 10% buffered formalin via the left ventricle for 4 minutes. The heart and proximal aorta were excised and the apex and lower half of the ventricles were removed. The remaining specimen was embedded in OCT (Tissue-Tek, Fisher Scientific), frozen on dry ice, and stored at \(-80^\circ\text{C}\) until sectioning. Serial cryosections were prepared through the ventricle until the aortic valves appeared. From then on, every fifth 10-\(\mu\text{m}\) section was collected on poly-D-lysine–coated slides until the aortic sinus was completely sectioned. Sections were stained with hematoxylin and Oil Red O, which specifically stains lipids. Slides were examined by light microscopy and atherosclerotic lesion area was quantified with computer assisted image analysis (Image-Pro Plus, Media Cybernetics, Bethesda, MD) as previously described \textsuperscript{5}, and averaged over 40 sections \textsuperscript{6}.

En Face Analysis of Aortas

The aorta, including the ascending arch, thoracic, and abdominal segments, was dissected, gently cleaned of the adventitia, and stained with Sudan IV \textsuperscript{5}. The surface lesion area was quantified with computer assisted image analysis (Image-Pro Plus, Media Cybernetics) as previously described \textsuperscript{5}.

Calcification Quantification

Calcified areas can be seen clearly in the hematoxylin stained sections, which was verified by Von Kossa calcium stain. The calcification area from each analyzed section was quantified in \(\mu\text{m}^2\) using computer assisted image analysis (Image-Pro Plus, Media Cybernetics).
The calcified areas per animal were added and multiplied with 10, corresponding to the thickness of the sections, which was 10 \( \mu \text{m} \). The total volume in \( \mu \text{m}^3 \) was used as the measurement of calcification for calcification. Total calcium in lyophilized aortic tissue was determined as previously described \(^7\).

**Immunoblotting**

Immunoblotting was performed as previously described \(^8\). Equal amounts of cellular protein or culture medium were used. Blots were incubated with specific antibodies to pSMAD1/5/8 or pSMAD2/3 (both 400 ng/ml; Cell Signaling Technology, Danvers, MA), ICAM-1 (100 ng/ml; Cell Signaling Technology), total SMAD, BMP-2, BMP-4, ALK1, ALK2, Chordin, VCAM-1 or E-selectin (all 200 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA), IL-6 (1 \( \mu \text{g/ml} \); R&D Systems, Minneapolis, MN), CV2 (400 ng/ml; R&D Systems), CD68 (1 \( \mu \text{g/ml} \); Dako, Carpinteria, CA), HSP70 (1 \( \mu \text{g/ml} \); Assay Designs, Ann Arbor, MI), or Noggin (400 ng/ml; Abcam, Cambridge, MA). \( \beta \)-Actin (1:5000 dilution; Sigma) was used as loading control. For optimal detection of VEGF in culture media, VEGF was first immunoprecipitated with anti-VEGF antibodies (Santa Cruz Biotechnology), as previously described \(^9\) and then analyzed by immunoblotting using specific antibodies to VEGF (200 ng/mL; R&D Systems). Densitometry using NIH Image J64 (http://rsb.info.nih.gov/nih-image/) was performed to compare protein levels.

**Validation of Antibodies**

The specificity of all antibodies was verified prior to use for immunoblotting and immunostaining (Supplemental Fig. I). For positive controls, commercially available antigens were obtained for BMP-2, BMP-4, VEGF, Noggin, IL-6 (all from R&D Systems), and HSP70 (Assay Designs). Mouse macrophages were used for CD68, and BMP-4 treated HAEC were used for ALK2, ALK1, ICAM1, VCAM-1, pSMAD1/5/8, pSMAD2/3 and total SMAD. We previously showed that siRNA to ALK2, ALK1, SMAD1 and SMAD2 transfected into HAEC depletes the signal on immunoblotting using the above antibodies \(^10\). In addition, the specificity of the anti-ALK1 antibodies was previously confirmed using HA-tagged ALK1 and anti-HA antibodies \(^11\). ICAM-1 and VCAM-1 are well known adhesion molecules in endothelial cells \(^12\).

**RNA Analysis**

Total RNA was isolated using the RNaseasy kit as per the manufacturer’s instruction (Qiagen, Valencia, CA). Real-time PCR assays were performed as previously described \(^9\) using an Applied Biosystems 7700 sequence detector (Applied Biosystems, Foster City, CA). Briefly, 2 \( \mu \)g of total RNA was reverse-transcribed with random hexamers using an MMLV Reverse Transcription Reagents kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. Each amplification mixture (20 \( \mu l \)) contained 25 ng of reverse-transcribed RNA, 8 \( \mu M \) forward primer, 2 \( \mu M \) reverse primer, 2 \( \mu M \) dual-labeled fluorogenic probe (Applied Biosystems), and 10 \( \mu l \) of Universal PCR mix Quantitect probe RT-PCR kit (Qiagen). PCR thermocycling parameters were 50 \(^\circ\)C for 2 min, 95 \(^\circ\)C for 10 min, and 40 cycles of 95 \(^\circ\)C for 15 s and 60 \(^\circ\)C for 1 min. All samples were analyzed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in parallel in the same run. Results of the real-time PCR data were represented as \( C_t \) values, where \( C_t \) is defined as the threshold cycle of PCR at which amplified product was first detected. To compare the different RNA samples in an experiment, we used the comparative \( C_t \) method \(^13, 14\) and compared the RNA expression in samples to that of the control in each experiment. The primers and probes were constructed so that the dynamic range of both the targets and the GAPDH reference were similar over a wide range of dilutions (1:1–10,000). PCR was performed as quadruplicates for each sample. The results were expressed as mean \( \pm \) S.D. for the relative expression levels compared with the control, and minimum values of four independent experiments were performed. Pre-designed primers and probes for mouse BMP-2, BMP-4,
MGP, ALK1, ALK2 and VEGF were obtained from Applied Biosystems as part of Taqman® Gene Expression Assays.

**Immunohistochemistry and Immunofluorescence**

Tissue sections were fixed in 4% paraformaldehyde and processed as previously described. For immunohistochemistry or immunofluorescence, sections were permeabilized with 0.5% Triton X-100 for 10 minutes, followed by 3 washes with wash buffer (WB, phosphate-buffered saline (PBS) containing 0.1% Tween-20). Non-specific antibody binding sites were blocked by incubating the sections for 30 minutes in blocking buffer (1% BSA, 2% goat serum and 0.5% Triton X-100 in PBS). Primary antibodies were diluted in antibody buffer (PBS containing 1% BSA, 0.5% Triton X-100), and sections were incubated for 60 minutes at room temperature, followed by several washes in WB. Alexa Fluor 488-conjugated (green fluorescence) or Alexa Fluor 594-conjugated (red fluorescence) secondary chick anti-goat or anti-rabbit antibodies (Molecular Probes, Eugene, OR) were applied to the sections and incubated for 30 minutes at room temperature. After several washes in WB and a brief equilibration of the sections with PBS, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). A DAPI stock solution was diluted to 300 nM in PBS, and 300 µl of the diluted solution was added to the sections, making certain that they were completely covered. The sample was incubated for 1-5 minutes and rinsed several times in PBS. Staining without primary antibodies served as controls. Images were acquired with an inverted Zeiss Axiovert 200 microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY, USA). To eliminate the possibility of false colocalization caused by emission filter bleed through, only images showing signals that were clearly visible by eye through the microscope when using the appropriate filters for the respective antibodies were considered significant and included in the results.

For immunostaining, we used specific antibodies to MGP (provided by Dr. Reidar Wallin, Wake Forest University, Winston-Salem, NC), pSMAD1/5/8, pSMAD2/3, ICAM-1 (all from Cell Signaling Technology), total SMAD, BMP-2, BMP-4, ALK1, ALK2, Chordin, VCAM-1, E-selectin (all from Santa Cruz Biotechnology), vWF (Invitrogen, Carlsbad, CA), VEGF, IL-6, CV2 (all from R&D Systems), CD68 (Dako), Noggin (Abcam), and HSP70 (Assay Designs).

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Serum levels of BMP-2 and -4 were determined by Quantikine® ELISA (R&D Systems) as per manufacturer’s instructions, using 100 µl of serum per assay.

**Complete Blood Count (CBC)**

Standard CBC was determined using a HemaTrue Hematology Analyzer (Heska Lab Systems, Loveland, CO) as per manufacturer’s instructions.

**Statistical Analysis**

Data were analyzed for statistical significance by ANOVA with post hoc Tukey’s analysis, or by the Mann-Whitney rank sum test or the unpaired Student t-test for unequal variance. The analyses were performed using the GraphPad Instat® 3.0 software (GraphPad Software, San Diego, CA). P-values less than 0.05 were considered significant.
Positive controls for antibodies used for immunoblotting and immunostaining

Immunoblotting was performed using positive controls obtained as indicated in the figure. The locations of a relevant weight marker is indicated in each blot.
**Supplemental Figure II**

Fat-feeding enhances and increased MGP expression limits aortic BMP-signaling. Apoe<sup>−/−</sup> and MGP<sup>lg/wt</sup> Apoe<sup>−/−</sup> mice fed chow (CH) or Western (HF) diet were examined. (G) BMP activity in aortic wall without lesions as determined by pSMAD1/5/8 immunohistochemistry. PSMAD2/3 and total SMAD are shown for comparison.
Supplemental Figure III

Expression of BMP inhibitors in Apoe<sup>−/−</sup> and MGP<sup>tg/wt</sup>;Apoe<sup>−/−</sup> mice fed chow or Western diet. (A-C) Aortic expression of CV2, Noggin and Chordin in Apoe<sup>−/−</sup> and MGP<sup>tg/wt</sup>;Apoe<sup>−/−</sup> mice fed chow (CH) or Western (HF) diet, as determined by real-time PCR (A), immunoblotting with densitometry (B), and immunofluorescence (C). Asterisks indicate statistically significant differences. **<0.01, ***<0.001, Tukey’s test.
Supplemental Figure IV

Increased MGP expression suppresses inflammatory activity

Aortic expression of CD68, IL-6, HSP70, ICAM-1, VCAM-1 and E-selectin in Apoe^-/- and MGP_tg/wt; Apoe^-/- mice a Western (HF) diet, as determined by real-time PCR and normalized to lesion area. The lesion areas used were derived from the en face analysis; 8.8% was used for the Apoe^-/- mice and 4.2% was used for the MGP_tg/wt; Apoe^-/- mice.

Asterisks indicate statistically significant differences. **<0.01, ***<0.001, Tukey’s test.

Online Figure IV
Supplemental Figure V

*Increased aortic BMP-signaling and expression of ALK1 and VEGF in MGP<sup>−/−</sup> mice.*

(A,B) BMP activity in aortas of Apoe<sup>−/−</sup> mice as determined by immunofluorescence (A) and immunoblotting with densitometry for pSMAD1/5/8 (B).

Structural changes in aortas of MGP<sup>−/−</sup>;Apoe<sup>−/−</sup> mice are visible in the H&E-stained sections (A, top).
Supplemental Figure VI

Expression of BMP inhibitors in Apoe<sup>−/−</sup> and MGP<sup>−/−</sup>;Apoe<sup>−/−</sup> mice.

(A-C) Aortic expression of CV2, Noggin and Chordin in Apoe<sup>−/−</sup> and MGP<sup>−/−</sup>;Apoe<sup>−/−</sup> mice, as determined by real-time PCR (A), immunoblotting with densitometry (B), and immunofluorescence (C). Asterisks indicate statistically significant differences. ***<0.001, Tukey’s test.
Online Table I.
Body mass and lipid levels of Apoe<sup>−/−</sup>, Apoe<sup>−/−</sup>;MGP<sup>tg/wt</sup>, and Apoe<sup>−/−</sup>;MGP<sup>−/−</sup> mice.

<table>
<thead>
<tr>
<th></th>
<th>Mice</th>
<th>Body Mass (gram)</th>
<th>Triglycerides (mg/dl)</th>
<th>Total Cholesterol (mg/dl)</th>
<th>HDL-C (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>After 16 weeks on Western diet</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>35.7±1.4</td>
<td>67.6±5.4</td>
<td>1534.2±219.9</td>
<td>14.4±3.2</td>
</tr>
<tr>
<td></td>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;;MGP&lt;sup&gt;tg/wt&lt;/sup&gt;</td>
<td>35.4±1.6</td>
<td>63.5±30.0</td>
<td>1332±177.3</td>
<td>16.4±2.0</td>
</tr>
<tr>
<td>female</td>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>27.9±1.3</td>
<td>63.8±27.2</td>
<td>1110.6±184</td>
<td>21.4±5.1</td>
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<tr>
<td></td>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;;MGP&lt;sup&gt;tg/wt&lt;/sup&gt;</td>
<td>28.2±0.7</td>
<td>60.5±25.3</td>
<td>1376.2±205.0</td>
<td>22.4±10.2</td>
</tr>
<tr>
<td><strong>After 18 weeks on chow diet</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>26.9±1.4</td>
<td>39.8±17.1</td>
<td>355.5±59.2</td>
<td>21±8.9</td>
</tr>
<tr>
<td></td>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;;MGP&lt;sup&gt;−/−&lt;/sup&gt; ***</td>
<td>17.2±7.0</td>
<td>33±11.8</td>
<td>428.8±53.9</td>
<td>20.6±7.1</td>
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<tr>
<td>female</td>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>23.7±0.8</td>
<td>34.5±9.2</td>
<td>339.3±51.2</td>
<td>17.8±9.4</td>
</tr>
<tr>
<td></td>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;;MGP&lt;sup&gt;−/−&lt;/sup&gt; ***</td>
<td>15.1±1.4</td>
<td>39.8±16.1</td>
<td>366.8±52.0</td>
<td>21.8±6.4</td>
</tr>
</tbody>
</table>

Asterisks indicate statistically significant differences. ***<0.001, Tukey’s test (n=3 for each group).
Online Table II.
Serum phosphate levels of Apoe\textsuperscript{−/−}, Apoe\textsuperscript{−/−};MGP\textsuperscript{tg/wt}, and Apoe\textsuperscript{−/−};MGP\textsuperscript{−/−} mice.

<table>
<thead>
<tr>
<th></th>
<th>Mice</th>
<th>Phosphate (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>After 16 weeks on</strong></td>
<td><strong>chow</strong></td>
<td></td>
</tr>
<tr>
<td>Chow diet</td>
<td>Apoe\textsuperscript{−/−}</td>
<td>0.782±0.027</td>
</tr>
<tr>
<td></td>
<td>Apoe\textsuperscript{−/−};MGP\textsuperscript{tg/wt}</td>
<td>0.783±0.031</td>
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<tr>
<td>Western diet</td>
<td>Apoe\textsuperscript{−/−}</td>
<td>0.779±0.022</td>
</tr>
<tr>
<td></td>
<td>Apoe\textsuperscript{−/−};MGP\textsuperscript{tg/wt}</td>
<td>0.786±0.022</td>
</tr>
<tr>
<td><strong>After 18 weeks on</strong></td>
<td><strong>chow diet</strong></td>
<td></td>
</tr>
<tr>
<td>Chow diet</td>
<td>Apoe\textsuperscript{−/−}</td>
<td>0.785±0.017</td>
</tr>
<tr>
<td></td>
<td>Apoe\textsuperscript{−/−};MGP\textsuperscript{−/−}</td>
<td>0.786±0.018</td>
</tr>
</tbody>
</table>

No statistically significant differences (n=3 for each group).
Online Table III.
Complete blood count (CBC) of Apoe\(^{-/-}\) and Apoe\(^{-/-}\);MGP\(^{-/-}\) mice.

<table>
<thead>
<tr>
<th>Mice</th>
<th>WBC</th>
<th>Lym</th>
<th>Mono</th>
<th>Gran</th>
<th>Lym %</th>
<th>Mono %</th>
<th>Gran %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoe(^{-/-})</td>
<td>5.27±0.32</td>
<td>4.17±0.23</td>
<td>0.53±0.06</td>
<td>0.57±8.9</td>
<td>79.57±0.72</td>
<td>8.50±0.62</td>
<td>11.93±0.15</td>
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<tr>
<td>Apoe(^{-/-});MGP(^{-/-})</td>
<td>5.43±0.12</td>
<td>4.27±0.15</td>
<td>0.57±0.06</td>
<td>0.60±0.01</td>
<td>79.17±0.71</td>
<td>8.77±0.58</td>
<td>12.07±0.45</td>
</tr>
</tbody>
</table>

No statistically significant differences (n=3 for each group).
REFERENCES