Regulation of Bone Morphogenetic Protein-4 by Matrix GLA Protein in Vascular Endothelial Cells Involves Activin-like Kinase Receptor 1*

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Matrix GLA protein (MGP) has previously been shown to enhance expression of vascular endothelial growth factor (VEGF) through the activin-like kinase receptor 1 (ALK1) in bovine aortic endothelial cells. MGP has also been identified as an inhibitor of bone morphogenetic protein-2 (BMP-2). This study showed that the effect of MGP on ALK1 signaling and VEGF expression in bovine aortic endothelial cells was dose-dependent, that a progressive increase of MGP levels ceased to be stimulatory and instead turned inhibitory. We identified a new regulatory pathway involving BMP that may explain this response. BMP-2 and BMP-4 induced expression of ALK1 in a dose-dependent fashion as determined by real-time PCR and immunoblotting. Activation of ALK1 signaling induced expression of MGP in addition to that of VEGF, allowing for negative feedback regulation of BMP by MGP. MGP inhibited BMP-4 activity similarly to that of BMP-2 and interacted with BMP-4 on a protein level as determined by co-immunoprecipitation. The dose-dependent effect on ALK1 expression and the stimulation of MGP and VEGF expression were dependent on signaling by transforming growth factor-β (TGF-β) and ALK1. Inhibition of TGF-β by neutralizing antibodies abolished the inhibitory effect of high BMP-4 levels on ALK1 expression and the induction of MGP and VEGF. Depletion of ALK1 by small interfering RNA abolished the induction of MGP and VEGF. MGP promoter activity was also stimulated by BMP-4 in a TGF-β-dependent fashion. The results suggest that the effects of BMP on endothelial cells occur in part through induction of ALK1, an effect that may be limited by ALK1-induced MGP.

Matrix GLA protein (MGP) is considered to be an inhibitor of cardiovascular calcification based on the extensive arterial calcification observed in MGP null mice (1, 2) and the arterial and valvular calcification detected after warfarin intake (3, 4). However, MGP is also expressed in vascular endothelium (5, 6), where its role is less well understood. We have previously shown that MGP enhances VEGF expression in bovine aortic endothelial cells (BAEC) by increasing the activity of transforming growth factor-β1 (TGF-β1) signaling through the activin-like kinase receptor 1 (ALK1) and the SMAD1/5/8 pathway (7). Other studies show that expression of MGP is increased in tubular endothelial cells (8), suggesting that MGP might be connected to resolution of angiogenesis.

ALK1 is a TGF-β type I receptor associated with angiogenesis (9). It activates the SMAD1/5/8 pathway similarly to BMP receptors, although its only known ligands are TGF-β1 and -β3 (10). Deficiency of ALK1 results in abnormal vascular formation with defects in the smooth muscle cell recruitment and arterio-venous malformations resembling human hereditary hemorrhagic telangiectasia (11, 12). TGF-β is known to have a biphase effect on angiogenesis (13), with low and high levels of TGF-β having a stimulatory and inhibitory effect, respectively. TGF-β is also known to induce MGP expression in endothelial and other cell types (7, 14).

Bone morphogenetic proteins (BMP), also members of the TGF-β superfamily of growth factors, have been shown to stimulate vasculogenesis and angiogenesis (15). BMP-4 is necessary for induction of mesoderm and endothelial progenitor cell differentiation (16). BMP-2 induces tumor angiogenesis (17, 18), and BMP-4 enhances VEGF secretion by human retinal pigment epithelial cells (19). Furthermore, BMP-2, -4, and -6 stimulate VEGF secretion in preosteoblasts, and BMP-2 and -6 modulate capillary tube formation (20, 21). Conversely, vascular formation is also affected by BMP inhibitors, including chordin, noggin, and the BMP-binding endothelial cell precursor-derived regulator (BMPER) (15, 22). We and others have shown that MGP binds and inhibits BMP-2 (23, 24), and we hypothesized that MGP may affect endothelial cells through BMP inhibition.

Our study shows that the effect of MGP on TGF-β1/ALK1 signaling and VEGF expression is dose-dependent in BAEC and that a progressive increase of MGP levels ceases to be stimula-
Regulation of BMP-4 by MGP Involves ALK1

tory and turns inhibitory. We have identified a new regulatory pathway that may contribute to or explain this response. Our results show that BMP-2 and BMP-4 induce the expression of ALK1, which, when activated, induces expression of MGP and VEGF. Inhibition of either TGF-β or ALK1 abolishes BMP-induced expression of MGP and VEGF. MGP enhances ALK1 signaling, thereby providing positive feedback. However, MGP also binds and inhibits BMP-2 and BMP-4, thereby providing negative feedback for BMP signaling, which ultimately leads to a decline in ALK1 expression and activity. Together, this may lead to a differential effect on ALK1 signaling. Our findings suggest that the effects of BMP on endothelial cells and angiogenesis occur in part through induction of ALK1, an effect that may be limited by ALK1-induced MGP.

MATERIALS AND METHODS

Cell Culture and Transfection Assays—BAEC were purchased from VEC Technologies, Inc. (Rensselaer, NY) and cultured as described previously (7). The BAEC were used between passages 2 and 15, except for transfection experiments when they were used between passages 2 and 6. Transient transfections of BAEC were performed with FuGENE 6 reagent (Roche Applied Science) and 500 ng of DNA/well as described previously for 24-well plates (7) and adjusted as needed for larger wells. The total amount of DNA used for each transfection was kept constant with parental expression vector. Recombinant human TGF-β1, BMP-2, BMP-4, and neutralizing antibodies to TGF-β1 (all from R&D Systems) were added at the time of transfection or plating. Luciferase assays were performed as described previously (7), with normalization to β-galactosidase or Renilla (Promega). Cell viability was determined by the trypan blue exclusion test as described previously (25) after treatment with BMP-2, BMP-4, TGF-β1, and N-terminally FLAG-tagged MGP (N-FLAG-MGP). Cells floating in the original medium were included in the test, and the result was given as the percentage of nonviable cells.

Inhibition of ALK1 by siRNA—Human aortic endothelial cells (HAEC) were used for siRNA experiments since the bovine ALK1 sequence was not available for siRNA design. The HAEC were cultured as described previously (26). Transient transfections of HAEC were performed with Lipofectamine-2000 (Invitrogen) using 60 nM siRNA; the amount of siRNA had been optimized as per the manufacturer’s instructions. Three separate ALK1 siRNAs (Silencer® predesigned siRNA, Ambion), and scrambled siRNA with the same nucleotide content were tested. When compared with unrelated control siRNA and scrambled siRNA, the ALK1 siRNA resulted in a 80–95% decrease in ALK1 mRNA and protein levels as determined by real-time PCR and immunoblotting, respectively. The siRNA that provided the most efficient inhibition (90–95%) was used for experiments.

Vector Constructions—The ALK1- and BMP-responsive luciferase reporter gene (BRE-Luc) was obtained from Dr. Peter ten Dijke, The Netherlands Cancer Institute, Amsterdam, The Netherlands(27). The construct for constitutively active ALK1 was obtained from Dr. Karen Lyons, UCLA. To construct the N-terminally FLAG-tagged human MGP (hMGP) vector, a fragment containing the coding region for hMGP was amplified by PCR. The FLAG tag was placed in the N terminus of the secreted, mature protein by subcloning of a synthesized FLAG-coding DNA fragment between the coding regions for the signal peptide and the mature protein. The FLAG-containing hMGP DNA fragment was amplified by PCR and subcloned into the Nhel and Xhol sites of pcDNA3.1(+)(Invitrogen) using restriction sites in the primers. The human MGP luciferase reporter genes (28) were obtained from Dr. Roland Schüle, University of Freiburg, Germany.

RNA Analysis—Total RNA was isolated from tissues or cultured cells using the RNaseasy kit (Qiagen). Real-time PCR assays were performed as described previously (7). The following primers and probes were used: human MGP (hMGP) forward (5’-GGGAAGCTTTGATGAATGATGACCTTCAA-3’), hMGP reverse (5’-CGATTAGGGCCAGCTGATTG-3’), and hMGP Taqman probe (FAM-TCTCTGCACCTTCTGCCGATGCC-TAMRA). Cells floating in the original medium were included in the test, and the result was given as the percentage of nonviable cells.

Immunoblotting—Immunoblotting was performed as described previously (7). Equal amounts of cellular protein or equal volumes of culture medium were compared. Blots were incubated with specific antibodies to hemagglutinin (HA) (1 µg/ml; Cell Signaling), ALK1 (0.4 µg/ml; Santa Cruz Biotechnology, D-20), BMP-4 (4 µg/ml; Santa Cruz Biotechnology), and GAPDH (0.1 µg/ml; Santa Cruz Biotechnology).
FLAG (2.5 μg/ml; Sigma), VEGF (2 μg/ml; Santa Cruz Biotechnology), or β-actin (0.6 μg/ml; Sigma).

Immunocytochemistry—BAEC were fixed in 4% paraformaldehyde for 10 min, and immunocytochemistry was performed as described previously (24). Rabbit polyclonal antibodies to MGP were provided by Dr. Reidar Wallin, Wake Forest University, Winston-Salem, NC. ABC reagents (Vector Laboratories) were used to detect antibody binding.

Immunoprecipitation—Co-immunoprecipitation of BMP-4 and N-FLAG-MGP was performed as described previously (24). BMP-4 (100 ng) was added to 1 ml of culture medium from BAEC collected 24 h after transfection of the N-FLAG-MGP construct or empty plasmid. Immunoprecipitation was performed using anti-BMP-4 antibodies (2 μg/ml; Santa Cruz Biotechnology), and immunoblotting was performed using anti-FLAG antibodies as described above. Alternatively, immunoprecipitation and immunoblotting were performed using anti-FLAG and anti-BMP-4 antibodies, respectively.

Quantification of N-FLAG-MGP—The concentration of N-FLAG-MGP in medium from BAEC transfected with the N-FLAG-MGP construct was determined using an enzyme-linked immunosorbent assay. N-terminal Met-FLAG-BAP™ fusion protein (Sigma) was used as a standard. To determine the N-FLAG-MGP concentration, 189 μl of sample (undiluted or diluted BAEC medium or standard protein dissolved in culture medium) was mixed with 21 μl of 10× coating buffer (1 M sodium carbonate, pH 9.6) and used for coating of 96-well plates (3 wells, 70 μl/well). After incubation for 1 h at 37 °C, remaining protein-binding sites were blocked with 200 μl/well of blocking buffer (phosphate-buffered saline (PBS) with 1% bovine serum albumin) and incubated for another 1 h at 37 °C. After repeated washes with PBS, the plates were ready for FLAG-protein determination. Fifty μl of anti-FLAG (1 μg/ml in PBS containing 5% (w/v) nonfat dry milk protein) was added to each well and incubated for 1 h at room temperature. After three washing cycles with PBS containing 0.05% Tween 20, the antibody bound to the plate was quantified by using a second antibody (goat anti-rabbit IgG conjugated with alkaline phosphatase (Santa Cruz Biotechnology; 0.4 μg/ml in PBS containing 5% (w/v) nonfat dry milk protein); 50 μl was added per well and incubated for 1 h at room temperature. After three washing cycles with PBS containing 0.05% Tween 20, the plates were stained with p-nitrophenyl phosphate liquid substrate system (Sigma), 150 μl/well, and read at 405 nm.

Statistics—Data were analyzed for statistical significance by analysis of variance with post hoc Scheffe’s analysis, unless otherwise stated. The analyses were performed using StatView, version 4.51 (Abacus Concepts). All experiments were repeated a minimum of three times.

FIGURE 2. MGP dose-dependently affects VEGF expression. A, MGP increases VEGF expression at low but not high levels of TGF-β1. BAEC were transfected with increasing amounts of N-FLAG-MGP construct and treated with TGF-β1 (0.5 or 4 ng/ml) for 24 h. VEGF expression was determined by real-time PCR and normalized to GAPDH expression. Secreted VEGF was visualized by immunoblotting of equal amounts of medium. B, MGP expression is increased after transfection with N-FLAG-MGP (human MGP) construct. Bovine and human MGP was determined in the same samples used in A by real-time PCR and normalized to GAPDH expression. Asterisks indicate statistically significant differences when compared with control (no hMGP plasmid) (*, p < 0.05; **, p < 0.001; ***, p < 0.0001; Scheffe’s test).
RESULTS

MGP Has Dose-dependent Effects on ALK1 Signaling Activity and VEGF Expression—We have previously shown that an increase in MGP levels enhances angiogenesis, ALK1 signaling, and VEGF expression (7). However, since MGP expression is high in tube-forming EC (8), it may also be connected with resolution of angiogenesis. To determine whether the effect of MGP on ALK1 signaling is dose-dependent, we co-transfected BAEC with a constant amount of the BRE-Luc reporter gene and increasing amounts of an expression construct, N-FLAG-MGP. The transfected cells were treated with TGF-β1 (0.5 ng/ml) for 24 h, after which the level of N-FLAG-MGP was determined in the medium by FLAG-enzyme-linked immunosorbent assay, and the luciferase activity was determined and normalized to β-galactosidase. The results showed that low levels of MGP stimulated ALK1 signaling up to 5-fold (5.06 ± 0.48) (Fig. 1), similarly to previous results (7), whereas high levels of MGP inhibited signaling. This suggested that the effect of MGP is dose-dependent in a biphasic fashion.

In our previous studies, we found that MGP-enhanced VEGF expression induced by 0.5 ng/ml TGF-β1 (7). Since 0.5 and 4 ng/ml TGF-β1 increases MGP expression about 2- and 6-fold, respectively, in BAEC (7), we predicted that combining MGP with 4 ng/ml TGF-β1 would no longer stimulate VEGF expression. To test this, we transfected BAEC with increasing amounts of N-FLAG-MGP construct and treated the cells with TGF-β1 (0.5 or 4 ng/ml). Cellular RNA was prepared after 24 h, and VEGF expression was determined by real-time PCR and normalized to GAPDH. Secreted VEGF was visualized by immunoblotting. The results showed that VEGF expression was only stimulated by MGP at the lower concentration of TGF-β1 (Fig. 2A), supporting that the effects of TGF-β1 and MGP are dose-dependent. Expression of human and bovine MGP, respectively, was determined by real-time PCR to confirm the transfection (Fig. 2B).

BMP-2 and BMP-4 Dose-dependently Induces Expression of ALK1, Which, When Activated, Induces Expression of MGP and VEGF—Since MGP is a BMP inhibitor that appears to regulate ALK1, we examined the effect of BMP-2 and BMP-4 on ALK1 expression. BAEC were treated for 24 h with BMP-2 or BMP-4 (0–100 ng/ml), and ALK1 expression was determined by real-time PCR and normalized to GAPDH. The results showed that both BMP-2 and BMP-4 dose-dependently increased ALK1 expression in a dose-dependent fashion, with initial stimulation followed by inhibition at high BMP-4 levels (Fig. 3, A and B). BMP-4 gave the most dramatic ALK1 induction (16.55 ± 2.08) and was therefore used in subsequent experiments. To verify that ALK1 protein also increased after BMP treatment, the cells were treated with BMP-4 (0–100 ng/ml) for 24 h. Cell lysates were prepared and analyzed by immunoblotting for ALK1. To verify that the ALK1 antibodies recognized the correct bands, we compared the ALK1 blots with blots from cells transfected with HA-tagged ALK1, which had been analyzed with anti-HA antibodies (Fig. 3C). Anti-HA antibodies recognized three bands, which were identical to the three bands recognized by the anti-ALK1 antibodies in BMP-4-treated cells. BMP-4 treat-

FIGURE 3. BMP dose-dependently induces expression of ALK1. BAEC were treated for 24 h with BMP-4 (0–100 ng/ml) (A) or BMP-2 (0–100 ng/ml) (B). ALK1 expression was determined by real-time PCR and normalized to GAPDH. C, HA-tagged ALK1 was transfected into BAEC and detected with anti-HA antibodies in cell lysates (left). The same bands were detected by the anti-ALK1 antibodies in ALK1 expressing BAEC (right). D, BAEC were treated for 24 h with BMP-4 (0–100 ng/ml). ALK1 expression was determined by immunoblotting with anti-ALK1 antibodies. Asterisks indicate statistically significant differences when compared with control (*, p < 0.05; ***, p < 0.0001; Scheffe’s test).
ment resulted in a similar dose-dependent increase in ALK1 protein as was seen for ALK1 mRNA (Fig. 3D).

We hypothesized that ALK1 induces MGP, which would be expected to enhance ALK1 activity (7) (Fig. 1) and also provide negative feedback for BMP. To determine whether activated ALK1 induces MGP expression in addition to that of VEGF, we transfected BAEC with an expression vector for constitutively active ALK1 (0–100 ng/well) in the presence of neutralizing anti-TGF-β antibodies (300 ng/ml) to block endogenous TGF-β activity. After 24 h, the expression of ALK1 was determined to verify transfection using real-time PCR with normalization to GAPDH expression and immunoblotting (A). Expression of MGP and VEGF was determined using real-time PCR and normalized to GAPDH expression (B). Secreted VEGF protein was visualized by immunoblotting, and MGP protein was visualized by immunocytochemistry (C). Asterisks indicate statistically significant differences when compared with control. (***, p < 0.0001; Scheffe’s test).

Cell viability was determined to assess for toxic the effects of BMP-2 (0–100 ng/ml), BMP-4 (0–100 ng/ml), TGF-β1 (0–4 ng/ml), and N-FLAG-MGP (0–100 ng/ml) using the trypan blue exclusion test. No significant change in cell viability was
Regulation of BMP-4 by MGP Involves ALK1

**A.**

N-FLAG-MGP

| N-FLAG-MGP (ng plasmid/well) | - 11 kDa |
|-----------------------------|---------|
| 0                           | N-FLAG-MGP |
| 20                          |          |
| 40                          |          |
| 60                          |          |
| 80                          |          |
| 100                         |          |

**B.**

Normalized Luciferase Activity (percent)

| N-FLAG-MGP (ng plasmid/well) | 0 | 10 | 20 | 40 | 60 | 80 | 100 |
|-------------------------------|---|----|----|----|----|----|-----|
| BMP2                          | 100|    |    |    |    |    |     |
| BMP4                          |      | 100|    |    |    |    |     |

**FIGURE 5.** MGP inhibits BMP-4 activity. BAEC were co-transfected with a constant amount of the BRE-Luc reporter gene and increasing amounts of an expression construct for N-FLAG-MGP. The cells were treated with BMP-2 (100 ng/ml) or BMP-4 (40 ng/ml). After 24 h of treatment, N-FLAG-MGP in the culture medium was visualized by immunoblotting with anti-FLAG antibodies (A), and luciferase activity was determined and normalized to β-galactosidase activity (B). Asterisks indicate statistically significant differences when compared with control. *, p < 0.05; ***, p < 0.0001; Scheffe’s test.

detected for any of the four factors at the concentrations used. The percentage of nonviable cells when combining all concentrations was 5.0 ± 0.5% for BMP-4, 5.3 ± 0.5% for BMP-2, 5.3 ± 0.4% for TGF-β1, and 5.4 ± 0.3% for N-FLAG-MGP.

**MGP Binds and Inhibits BMP-4**—Although BMP-4 is closely related to BMP-2, it has not been shown that MGP inhibits BMP-4 activity. To determine the effect of MGP on BMP-4 activity in comparison with that of BMP-2, BAEC were transfected with increasing amounts of N-FLAG-MGP expression construct and a constant amount of the BRE-Luc reporter gene and treated with BMP-2 (100 ng/ml) or BMP-4 (40 ng/ml) in the presence of neutralizing anti-TGF-β antibodies (300 ng/ml) to avoid the effects of TGF-β on endogenous MGP expression.

The BMP concentrations were the lowest concentrations that gave maximal signaling activity in our system (data not shown). After 24 h, N-FLAG-MGP was visualized in the medium by immunoblotting with anti-FLAG antibodies, and the luciferase activity was determined and normalized to β-galactosidase activity (Fig. 5A). The results showed that increased BMP levels progressively inhibited luciferase activity induced by both BMP-2 and BMP-4 (Fig. 5B).

To determine whether MGP interacts with BMP-4, we used BAEC medium that contained both N-FLAG-MGP and BMP-4. We immunoprecipitated with antibodies to BMP-4 or to FLAG in the FLAG-tagged MGP. The BMP-4 immunoprecipitate was analyzed by immunoblotting for N-FLAG-MGP using the anti-FLAG-antibodies, and the FLAG immunoprecipitate was analyzed by immunoblotting using the anti-BMP-4. The results showed that BMP-4 and N-FLAG-MGP co-precipitated in both cases (Fig. 6), suggesting that the two proteins interact. Interestingly, the anti-BMP-4 antibodies precipitated both high and low molecular weight forms of MGP detected in the medium. Other investigators have shown that the change in molecular weight of MGP is due to C-terminal truncation of seven amino acids (RKRRGDKT) (29). To confirm that the reported truncation does not abolish MGP binding, we repeated the co-immunoprecipitation experiments using a mutated form of N-FLAG-MGP lacking the seven amino acids. The results confirmed that BMP-4 and the truncated MGP co-precipitated (data not shown). Thus, the truncation does not abolish interaction with BMP.

**BMP-4 Effects on Expression of MGP and VEGF Are Dependent on TGF-β and ALK1**—We predicted that inhibition of ALK1 signaling and MGP induction would allow continued BMP-4 stimulation of ALK1 expression at high BMP-4 levels.

To test this, we first used neutralizing antibodies to TGF-β, the only known ALK1 ligand. (The BAEC expressed ~0.3–0.5 ng/ml TGF-β per 24 h at baseline.) We treated BAEC with BMP-4 (0–100 ng/ml) in the presence of anti-TGF-β antibodies (300 ng/ml) for 24 h and compared it with BMP-4 only treatments. The presence of anti-TGF-β antibodies did abolish the dose-dependent ALK1 response. Instead, expression of ALK1 continued to increase at high BMP-4 levels as determined by real-time PCR with normalization to GAPDH and ALK1 immunoblotting (Fig. 7A). Furthermore, we expected expression of MGP and VEGF to decrease when ALK1 signaling was inhibited. This was tested with real-time PCR with normalization to GAPDH. The results showed that expression of MGP and VEGF was abolished (Fig. 7B), which suggests that the BMP-4 effects on MGP and VEGF are TGF-β-dependent. The peak of BMP-4-induced ALK1 expression preceded that of MGP in these experiments. This may be a reflection of the ability of MGP to enhance ALK1 activity at low to intermediate levels (Fig. 1) (7), which would enhance MGP expression...
despite gradually declining ALK1 expression. Ultimately, MGP expression would also start to decline. However, we cannot exclude additional signaling mechanisms decreasing ALK1 expression and increasing MGP expression at high BMP-4 levels.

We further predicted that elimination of the ALK1 receptor would prevent BMP-4-induced expression of MGP and VEGF. Since the bovine ALK1 nucleotide sequence is not available, we used human aortic endothelial cells (HAEC), which express ALK1. The HAEC were transfected with ALK1 siRNA and compared with unrelated control siRNA or scrambled siRNA. Transfection of ALK1 siRNA resulted in >90% decrease in ALK1 mRNA levels after 24 h as determined by real-time PCR and immunoblotting, whereas the two control siRNAs did not affect ALK1 expression (Fig. 8A). HAEC transfected with ALK1 siRNA or control siRNA were then treated with BMP-4 (0–100 ng/ml) starting 24 h after transfection. After 24 h of treatment, expression of MGP and VEGF was determined by real-time PCR and normalized to GAPDH. The results showed that the ALK1 siRNA prevented BMP-4 induction of ALK1 expression (Fig. 8B) and abolished the stimulatory effect of BMP-4 on expression of both MGP and VEGF (Fig. 8C). Together, the results suggest that TGF-β and ALK1 mediates the effect of BMP-4 on MGP and VEGF expression.

The MGP Promoter Is Induced by BMP-4 in a TGF-β-dependent Fashion—To determine whether BMP-4 stimulates the MGP promoter and whether such stimulation is TGF-β-dependent, we used luciferase reporter genes containing 3.6 or 1.8 kb of the MGP promoter (28). Smad4 promoter elements involved in TGF-β signaling are predicted between 2.6 and 3 kb by the Genomatix Software and would only be present in the 3.6-kb promoter fragment. BAEC were transfected with the luciferase reporter genes and treated with BMP-4 (0–100 ng/ml) only or in the presence of TGF-β1 (0.5 ng/ml) or anti-TGF-β antibodies (300 ng/ml). After 24 h, luciferase activity was determined and normalized to Renilla. The results for the 3.6-kb MGP promoter fragment showed that BMP-4 only increased promoter activity 2-fold (2.18 ± 0.04), whereas the addition of TGF-β to the BMP-4 increased promoter activity 8-fold (8.42 ± 0.10) (Fig. 9A). Anti-TGF-β antibodies, on the other hand, decreased promoter activity by two-thirds (0.31 ± 0.02). The results for the 1.8-kb MGP promoter fragment showed that BMP-4 still had a mild effect on promoter activity (2.32 ± 0.11), whereas the addition of TGF-β to the BMP-4 only increased promoter activity 1.6-fold (1.61 ± 0.03). (Fig. 9B). Anti-TGF-antibodies decreased activity by about a fourth (0.77 ± 0.01). The results
suggest that BMP-4 activation of the MGP promoter is TGF-β-mediated in a similar way to MGP expression. They also support the presence of a TGF-β-responsive element in the 3.6-kb promoter fragment only. We cannot exclude that BMP-4 also affects the MGP promoter through other signaling pathways.

DISCUSSION

Our studies suggest that BMP-4, ALK1, and MGP are part of a regulatory circuit in vascular endothelium and that BMP-4 depends on TGF-β/ALK1 signaling to affect expression of MGP and VEGF. The regulatory circuit may in part explain the dose-dependent effect of MGP on TGF-β/ALK1 signaling and VEGF expression. Low to intermediate levels of MGP enhances ALK1 signaling and VEGF expression. MGP also provides a progressive negative feedback for BMP that ultimately curbs induction of ALK1 and subsequent VEGF expression (see proposed model, Fig. 10). This regulatory system may also explain the dose-dependent effect of TGF-β on angiogenesis (13) since TGF-β induces MGP expression in multiple cell types including EC.

The BMPs have been recognized for ability to stimulate vasculogenesis in the embryo and the adult (15). BMP-4 is necessary for endothelial progenitor cell differentiation (16). BMP-2 induces tumor angiogenesis (17, 18), and BMP-2 and -6 modulate capillary tube formation (20, 21). Induction of ALK1 expression provides a way for BMP to affect angiogenesis and enhance VEGF secretion as seen in cells treated with BMP-2, -4, and -6 (19, 20), provided that the appropriate ligand for ALK1 is present. So far, only TGF-β1 and -β3 have been shown to stimulate ALK1 signaling (10), but ALK1 may also depend on other, not yet identified ligands. BMP-4 has been identified as an inflammatory mediator induced by oscillatory shear stress in endothelial cells (30, 31), and it is possible that BMP-4 and ALK1 induction are involved in initiation of angiogenesis and remodeling of the vascular wall, seen in pathologies such as atherosclerosis, systemic and pulmonary hypertension, and arterial and valvular stenoses (32, 33). A dysregulation of MGP and VEGF expression might contribute to the pathogenesis of hereditary hemorrhagic telangiectasia in ALK1-deficiency, characterized by arterio-venous malformations and defective recruitment of smooth muscle cell precursor cells (11, 12).

Differences in MGP levels have been associated with different tumor characteristics. Less MGP was found in symptomatic lung tumors that were clinically detected than in asymptomatic tumors that were CT scan-detected (34). Reduced levels of MGP were also detected in colorectal adenocarcinomas when compared with normal tissue (35). Since high levels of MGP
have been associated with tubular EC (8) and may regulate ALK1 expression, it is possible that a reduction of MGP results in a deregulation of angiogenesis.

The dose dependence of BMP-induced ALK1 expression might be useful in developmental patterning involving BMP gradients (36). Garfinkel et al. (37) have also shown that BMP-2 and MGP are able to function as an activator-inhibitor pair that affects pattern formation in vascular mesenchymal cells as predicted by a mathematical model. ALK1 expression has been reported in vascular and pulmonary mesenchyme (38, 39) and might be important in the balance between BMP and MGP.

Our studies revealed that the inhibitory effect of MGP is not limited to BMP-2 but also includes BMP-4. MGP and BMP-4 co-precipitate from culture medium, similarly to MGP and BMP-2 (24). Furthermore, BMP-4 also co-precipitated with a short form of MGP that lacks 7 amino acids, generated by C-terminal truncation of MGP (28). It suggests that this particular modification of MGP does not regulate BMP-binding. However, it may affect localization or matrix association of BMP since the C-terminal of MGP has been implicated in binding to vitronectin (40). Our previous studies showed that MGP abolished binding of BMP-2 to cell surface receptors (24), but MGP might still affect the receptor complex by interfering with other proteins that are recruited to the complex such as accessory receptors.

Our results do not explain the stimulatory effect of low to intermediate levels of MGP on ALK1-induced VEGF expression (7) (Fig. 2). It is possible that MGP increases availability of ligand or type II receptor for ALK1. However, we have not found any significant differences in TGF-β expression during our experiments (data not shown), and the type II BMP receptor, which would be more available after an increase in BMP inhibition, has so far not been connected with ALK1 (41).

In summary, our studies have identified a new regulatory pathway involving BMP-2/4, ALK1, and MGP, allowing for a reciprocal regulation of BMP and MGP through ALK1 in a TGF-β-dependent fashion.

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