Matrix GLA Protein Stimulates VEGF Expression through Increased Transforming Growth Factor-β1 Activity in Endothelial Cells*

Received for publication, June 21, 2004, and in revised form, September 7, 2004
Published, JBC Papers in Press, September 27, 2004, DOI 10.1074/jbc.M406868200

Kristina Bostrom‡,§, Amina F. Zebboudj†, Yucheng Yao‡, Than S. Lin‡, and Alejandro Torres‡
From the ‡Division of Cardiology, David Geffen School of Medicine, and the §Molecular Biology Institute,
University of California, Los Angeles, California 90095-1679

Matrix GLA protein (MGP) is expressed in endothelial cells (EC), and MGP deficiency results in developmental defects suggesting involvement in EC function. To determine the role of MGP in EC, we cultured bovine aortic EC with increasing concentrations of human MGP (hMGP) for 24 h. The results showed increased proliferation, migration, tube formation, and increased release of vascular endothelial growth factor-A (VEGF-A) and basic fibroblast growth factor (bFGF). HMG, added endogenously or transiently expressed, increased VEGF gene expression dose-dependently as determined by real-time PCR. To determine the mechanism by which hMGP increased VEGF expression, we studied the effect of MGP on the activity of transforming growth factor (TGF)-β1 compared with that of bone morphogenetic protein (BMP)-2 using transfection assays with TGF-β1 and BMP-response element reporter genes. Our results showed a strong enhancement of TGF-β1 activity by hMGP, which was paralleled by increased VEGF expression. BMP-2 activity, on the other hand, was inhibited by hMGP. Neutralizing antibodies to TGF-β1 blocked the effect of MGP on VEGF expression. The enhanced TGF-β1 activity specifically activated the Smad1/5 pathway indicating that the TGF-β receptor activin-like kinase 1 (ALK1) had been stimulated. It occurred without changes in expression of TGF-β1 or ALK1 and was mimicked by transfection of constitutively active ALK1, which increased VEGF expression. Expression of VEGF and MGP was induced by TGF-β1, but the induction of MGP preceded that of VEGF, consistent with a promoting effect on VEGF expression. Together, the results suggest that MGP plays a role in EC function, altering the response to TGF-β superfamily growth factors.

Expression of matrix GLA protein (MGP)† has been reported in vascular endothelium of species as different as human and teleost fish (1, 2), suggesting that MGP has a role in endothelial cell (EC) function. In addition, several features in MGP deficiency in mice (3) and the human equivalent, Keutel syndrome (4–8), suggest involvement of the vascular endothelium. Peripheral pulmonary artery stenosis (4–8) may result from a failure in angiogenesis or fusion of peripheral and central pulmonary vessels during lung development (9, 10). Arterial calcification and replacement of smooth muscle cells by cartilage-like cells (3) may be due to endothelial dysfunction during vascular development (11). Loss of architecture and hypertrophic chondrocytes in the bone growth plate (3) may in part be due to disturbed invasion of EC (12). Furthermore, increased MGP expression has been reported in tube-forming EC as determined by subtractive hybridization (13), and in myometrial EC treated with vascular endothelial growth factor (VEGF) as determined by microarray analysis (14). Altered expression of MGP has also been reported in several types of malignancies, including glioma, ovarian cancer, colorectal adenocarcinoma, and urogenital malignancies (15–18) and may relate to vascularization of the tumors.

We have previously shown that MGP modulates the activity of bone morphogenetic protein-2 (BMP-2) (19–21), a member of the transforming growth factor (TGF)-β superfamily of growth factors critical for morphogenesis and bone formation (22–25). TGF-β1 is another member of the same family and is known to stimulate expression of VEGF in multiple cell types (26–28). Two distinct TGF-β signaling cascades have been identified within EC, the activin-like kinase 1 (ALK1)-Smad1/5 pathway, and the ALK5-Smad2/3 pathway. ALK1 expression is predominantly up-regulated in EC at sites of angiogenesis and appears to have a stronger involvement with the activation phase of angiogenesis, whereas ALK5 appears more involved with the resolution phase (29–31).

The aim of this study is to determine whether MGP affects EC function. Our results indicate that MGP promotes release of VEGF-A and basic fibroblast growth factor (bFGF), both involved in angiogenesis. Furthermore, MGP increased VEGF gene expression through enhanced TGF-β1 activity without significant effect on expression of TGF-β1, ALK1, or ALK5. A concurrent inhibition of BMP-2 activity was detected. The enhanced TGF-β1 activity specifically activated the Smad1/5 pathway indicating that ALK1 had been stimulated by the presence of MGP. The effect on VEGF expression was blocked by neutralizing antibodies to TGF-β and mimicked by transfection of constitutively active ALK1, which also increased VEGF expression. Expression of VEGF and MGP were induced by TGF-β1, however, the induction of MGP preceded that of VEGF, consistent with a role for MGP in promoting VEGF expression. Together, the results suggest that MGP plays a role in EC function by altering the response to TGF-β growth factors, which in part may explain the vascular abnormalities seen in MGP deficiency.

* This work was funded in part by National Institutes of Health Grants HL04270 and HL030568, by the American Heart Association (National), and by the Laubisch Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Division of Cardiology, David Geffen School of Medicine at UCLA, Box 951679, Rm. 47-123 CHS, Los Angeles, CA 90095-1679. Tel.: 310-794-4417; Fax: 310-206-9133; E-mail: kbostrom@mednet.ucla.edu.
¶ This paper is available online at http://www.jbc.org

† The abbreviations used are: MGP, matrix GLA protein; EC, endothelial cell; VEGF, vascular endothelial growth factor; BMP-2, bone morphogenetic protein-2; TGF, transforming growth factor; ALK, activin-like kinase; bFGF, basic fibroblast growth factor; BAEC, bovine aortic endothelial cell; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; b, bovine; h, human; LAP, latency-associated peptide; caALK1, constitutively active ALK1.
MATERIALS AND METHODS

Cell Culture and Transfection Assays—Bovine aortic endothelial cells (BAEC) were purchased from VEC Technologies, Inc. (Rensselaer, NY). They were cultured in Dulbecco’s modified Eagle’s medium supplemented with 15% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), 100 units/ml penicillin, 100 units/ml streptomycin, 0.1% sodium pyruvate, and 2 mM L-glutamine (20°C) according to the manufacturer’s protocol. BAECs were purchased from VEC Technologies, Inc. (Rensselaer, NY).

Transient transfections of BAECs were performed in quadruplicates in 24-well culture dishes plated onto 24-well plates at 3 × 10⁴ cells/well 20–24 h prior to transfection. The cells were transiently transfected using 1.5 μl of FuGene6 reagent (Roche Molecular Biochemicals) and 500 ng of DNA per well (usually 200 ng of luciferase reporter constructs, 200 ng of various expression constructs, and 100 ng of β-galactosidase encoding construct as a control for transfection efficiency). The total amount of expression construct was kept constant with parental expression vector. Recombinant human TGF-β1/R&D Systems, Minneapolis, MN), bovine bFGF (R&D Systems), recombinant human VEGF (R&D Systems), human MGP or human BMP-2 were added at the time of transfection. Human MGP and BMP-2 were added in the form of conditioned medium, and the methods to prepare the media and determine the levels of MGP and BMP-2, respectively, have been described previously (21). In experiments where conditioned medium was used, the level of conditioned medium was kept constant at 80% of the total conditioned medium.

Cells were taken for analysis 24 h after transfection. For luciferase assays, the cells were lysed in 100 μl of Passive Lysis Buffer (Promega, Madison, WI) per well. The cells were freeze-thawed twice and agitation for 15 min. Two 20-μl and two 10-μl aliquots from each well were used for luciferase assays (Promega) and β-galactosidase assays, respectively. Luciferase activity was determined using an AutoLumat LB953 system and the reaction parameters. The following bovine primers and protocol to ensure suitability for the ABI Prism 7700 sequence detection system and the reaction parameters. The following bovine primers and protocol to ensure suitability for the ABI Prism 7700 sequence detection system and the reaction parameters. The following bovine primers and protocol to ensure suitability for the ABI Prism 7700 sequence detection system and the reaction parameters. The following bovine primers and protocol to ensure suitability for the ABI Prism 7700 sequence detection system and the reaction parameters. The following bovine primers and protocol to ensure suitability for the ABI Prism 7700 sequence detection system and the reaction parameters. The following bovine primers and protocol to ensure suitability for the ABI Prism 7700 sequence detection system and the reaction parameters. The following bovine primers and protocol to ensure suitability for the ABI Prism 7700 sequence detection system and the reaction parameters.
concentration of MGP 60 ng/ml) or the same amount of sham-conditioned medium, and 300 μl was added to each well of a 12-well plate and incubated at 37 °C for 30 min to allow polymerization. BAECs were suspended in the same EC medium containing MGP-conditioned medium (final concentration of MGP 60 ng/ml) at a density of 5 × 10^4 cells/well, and 400 μl of the cell suspension was added to each well of the plate and incubated for 48 h in the presence or absence of MGP.

Vector Constructions—The construct containing full-length hMGP cDNA has previously been described (19). The TGF-β-responsive p3TP-lux luciferase reporter gene has previously been described (34). The BMP-responsive luciferase reporter gene was obtained from Dr. Peter ten Dijke, The Netherlands Cancer Institute, Amsterdam, The Netherlands, and has previously been described (35). The construct for constitutively active ALK1 was obtained from Dr. Karen Lyons, University of California, Los Angeles, CA.

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, Berkeley, CA). All experiments were repeated a minimum of three times.

RESULTS

MGP Increases Release of Biologically Active VEGF and bFGF in BAEC—Increased MGP expression has previously been reported in tube-forming endothelial cells as determined by subtractive hybridization (13), and VEGF has been reported to increase MGP expression in myometrial EC (14). To determine whether MGP affects endothelial cell function, assays for cell proliferation, cell migration, and tube formation were performed.

Cell proliferation was determined using [3H]thymidine incorporation in quiescent BAECs exposed to medium from BAECs treated with hMGP (0–50 ng/ml) for 24 h. BAEC proliferation increased significantly after exposure to medium from hMGP-treated BAECs compared with medium exposed to control cells (hMGP 0 ng/ml). B, migration of BAECs increases after hMGP treatment. Cell migration was determined using the endothelial wounding assay in confluent monolayers of BAECs that had been treated for 24 h with hMGP (0–100 ng/ml) prior to wounding. Results showed a significant increase in migration after hMGP treatment. Asterisks indicate statistically significant differences compared with control (hMGP 0 ng/ml) (**, p < 0.01; ***, p < 0.001; Scheffe’s test).

C, BAEC tube formation on Matrigel was attenuated after hMGP treatment. BAECs were plated at the density 2 × 10^4 cells/well on Matrigel in 12-well plates. Medium containing hMGP (60 ng/ml) or control medium was added to the Matrigel, and the cells were incubated for 48 h. Original magnification, ×40.
monolayers of BAECs that had been treated for 24 h with MGP (0–100 ng/ml) prior to wounding. Results showed a significant increase in migration after hMGP treatment (Fig. 1B). Tube formation was assessed in Matrigel where cells were exposed to hMGP (60 ng/ml) or control medium for 24 h. Results showed an increase in tube formation in the presence of hMGP compared with control (Fig. 1C).

To determine the effect of MGP on levels of VEGF and bFGF, BAECs were treated with hMGP, at concentrations between 0 and 100 ng/ml. HMGP was added at the time of plating, and after 24 h VEGF and bFGF were determined in the medium and cellular extract using immunoblotting. To better visualize VEGF from the medium, VEGF was immunoprecipitated prior to immunoblotting. Results showed that hMGP stimulated the release of both VEGF-A (21 kDa) and bFGF into the medium, whereas the levels in the cell extracts remained constant or decreased (Fig. 2).

**MGP Increases Expression of VEGF**—To determine whether MGP also increased expression of VEGF, BAECs were treated with hMGP at concentrations between 0 and 100 ng/ml. HMGP was added at the time of plating, and after 24 h VEGF expression was determined using real-time PCR and normalized to GAPDH expression. Results showed a dose-dependent increase in VEGF expression (Fig. 3A), which increased ~4-fold after treatment with high levels of hMGP.

To determine whether overexpression of MGP using transient transfection would yield similar results to exogenous treatment with MGP, BAECs were transfected with an expression vector containing hMGP. Similar, but less pronounced, results were obtained in BAEC transiently transfected with hMGP. The typical transfection efficiency in BAEC was 10–20%, and VEGF expression increased 1.5- to 2-fold when determined 24 h after transfection (Fig. 3B). Corresponding increase of VEGF-A protein was detected in the medium 24 h after transfection (data not shown). To ensure that the transfection of the hMGP vector increased MGP expression and MGP levels in the medium, we performed real-time PCR for bovine and human MGP normalized to bovine GAPDH, and quantified total MPG in the medium. As expected, the results showed a large increase in human MGP expression after trans-
MGP Stimulates VEGF Expression through TGF-β1

FIG. 4. TGF-β1 and BMP-2 affects VEGF expression differently in BAEC. BAECs were treated for 24 h with TGF-β1 (0–8 ng/ml) or BMP-2 (0–64 ng/ml) after which VEGF expression was determined using real-time PCR and normalized to GAPDH expression. VEGF expression increases after TGF-β1 treatment and decreases after BMP-2 treatment. Asterisks indicate statistically significant differences compared with control (*, p < 0.05; ***, p < 0.0001; Scheffe's test).

MGP increases the luciferase activity about 25-fold, and the VEGF expression about 1.5-fold. However, transfecting hMGP plasmid in addition to the addition of 0.5 ng/ml TGF-β1 increases the luciferase activity up to 100-fold and the VEGF expression up to 4.5-fold. Thus, TGF-β1 exerts a stronger effect in the presence of MGP.

On the other hand, increasing expression of hMGP in BAECs treated with BMP-2 inhibited BMP-2 activity up to 60–70% at all levels of BMP-2 (Fig. 6). Again, MGP expression increased significantly after transfection with the hMGP construct similar to the results in Fig. 5C (data not shown).

Neutralizing Antibodies to TGF-β Abolish the Stimulating Effect of MGP on VEGF Expression—To confirm that the effect of MGP on VEGF expression can be blocked by inhibiting TGF-β signaling, neutralizing TGF-β antibodies were used. First, increasing amounts of TGF-β antibodies were added to BAECs, and VEGF expression was determined by real-time PCR and normalized to GAPDH expression. Results showed that VEGF expression decreased up to 40% in the presence of 50–600 ng/ml TGF-β antibodies (Fig. 7, left). BAECs were then treated with increasing amounts of hMGP (0–50 ng/ml). As described above, MGP dose-dependently increased VEGF expression (Fig. 7, middle). Finally, TGF-β antibodies were added at 300 ng/ml to BAECs treated with increasing amounts of hMGP (0–50 ng/ml). Results showed that the neutralizing TGF-β antibodies abolished the stimulatory effect of MGP on VEGF expression (Fig. 7, right), supporting the notion that MGP increases VEGF expression by acting through TGF-β1. These experiments indicate that the effect of MGP in the absence of exogenous TGF-β1 is probably due to endogenous TGF-β1 expression. TGF-β1 expression is detected by real-time PCR (Fig. 8A), and the TGF-β1 protein is detected by immunoblotting (data not shown).

MGP Does Not Affect Expression of TGF-β1, ALK1, or ALK5 or Protein Levels of TGF-β1 LAP—To determine whether changes in TGF-β1, TGF-β1 LAP, ALK1, or ALK5 would explain the increased TGF-β1 activity in presence of MGP, BAECs were treated with hMGP at concentrations between 0 and 100 ng/ml, or transfected with increasing amount of hMGP expression construct. After 24 h, TGF-β1 expression was determined using real-time PCR and normalized to GAPDH expression. Results showed little effect of hMGP on TGF-β1 expression in both hMGP-treated and hMGP-transfected cells (Fig. 8A). MGP expression increased significantly after transfection with the hMGP construct, similar to results in Figs. 3B and 5C (data not shown). Expression of ALK1 and ALK5 was determined by semi-quantitative RT-PCR using primers based on human ALK1 and ALK5 (the bovine receptors have not been sequenced) and normalized to GAPDH. Results showed virtually no effect on ALK1 or ALK5 expression in hMGP-treated cells (Fig. 8B). We also determined the TGF-β1 latency-associated peptide (LAP) (37) by immunoblotting the medium after hMGP treatment, using an antibody raised against human TGF-β1 LAP. Changes in TGF-β1 activation would be reflected in the levels of TGF-β1 LAP. However, the results showed little effect on protein levels of TGF-β1 LAP in the hMGP-treated cells (Fig. 8C). Together, these results do not explain the increased TGF-β1 activity, suggesting that MGP affects receptor-ligand interactions.

Stimulation of MGP Expression Precedes That of VEGF Expression after TGF-β1 Treatment—TGF-β1 has been reported to stimulate both VEGF and MGP expression (26–28, 38–40). In Fig. 4 we showed that TGF-β1 does increase VEGF expression in BAECs after 24 h of treatment. To determine if TGF-β1 also stimulates MGP expression, BAECs were treated with TGF-β1 (0–6 ng/ml) for 24 h, after which MGP expression was not detected by immunoblotting (data not shown).
FIG. 5. A, MGP stimulates TGF-β1 activity at constant levels of TGF-β1. A TGF-β-responsive luciferase reporter gene construct was co-transfected into BAEC together with increasing amounts of hMGP expression construct (transfection efficiency 10–20%), and the cells were subsequently incubated in the absence of exogenous TGF-β1, or with TGF-β1 (0.25 or 0.5 ng/ml). The luciferase activity was determined after 24 h of TGF-β1 treatment, and was normalized to β-galactosidase activity. The inset is an enlargement of the bars on the left, with no added TGF-β1. B, human MGP increases VEGF expression at constant levels of TGF-β1. BAECs were transfected as described under A, and VEGF expression was determined in cells incubated in absence of exogenous TGF-β1, or with 0.5 ng/ml TGF-β1 by real-time PCR and normalized to GAPDH expression. The VEGF expression increased in parallel with the TGF-β1 activity as determined in A. C, MGP expression increased after transfection with hMGP construct. Bovine and human MGP was determined in the same samples used in B by real-time PCR and normalized to GAPDH expression. Asterisks indicate statistically significant differences compared with control (no hMGP plasmid) for each TGF-β1 concentration (*, p < 0.05; **, p < 0.001; ***, p < 0.0001; Scheffe’s test).
determined using real-time PCR and normalized to GAPDH expression. The results showed that TGF-β1 significantly increased MGP expression (Fig. 9A). Because both TGF-β1 and MGP stimulated VEGF expression, and TGF-β1 also stimulated MGP expression, we treated BAECs with 0.5 ng/ml TGF-β1 and determined VEGF and MGP expression by real-time PCR at different time points following the start of the treatment. The results of this time course showed that MGP expression preceded VEGF expression (Fig. 9B), and thus, were consistent with a role for MGP in supporting VEGF expression.

MGP Treatment Activates Smad1/5—TGF-β1 activates the ALK1-Smad1/5 and ALK5-Smad2/3 pathway in EC (29–31). ALK1 appears to have a stronger involvement with the activation phase of angiogenesis, whereas ALK5 appears more involved with the resolution phase. Both receptors are expressed in our cells (Fig. 8C). To determine whether MGP activates Smad1/5 or Smad2/3, we treated BAEC without MGP or with 60 ng/ml hMGP for time periods between 1 min and 4 h. At each time point, cell lysates were prepared and immunoblotting for P-Smad1/5, P-Smad2/3, and total Smad was performed. The results showed a clear Smad1/5 activation after 1 h in samples treated with MGP compared with control cells (Fig. 10). No activation or a mild inhibition of Smad2/3 was detected. This suggests that MGP selectively promotes TGF-β signaling through ALK1.

Constitutively Active ALK1 Mimics the Effect of MGP on VEGF Expression—To determine whether constitutively active (ca)ALK1 would mimic the effect of MGP on VEGF expression, we transfected the BAEC with a caALK1 construct (transfection efficiency 10–20%). The VEGF expression was determined 24 h after transfection by real-time PCR and normalized to GAPDH. The results showed that caALK1 increased VEGF expression significantly (Fig. 11A), thus mimicking the effect of MGP. Real-time PCR for ALK1 normalized to GAPDH was performed to ensure that the ALK1 concentration increased after transfection with caALK1 construct (Fig. 11B).
MGP Stimulates VEGF Expression through TGF-β1

FIG. 8. A, TGF-β1 expression in BAEC does not increase after treatment or transfection with hMGP. BAECs were transfected with hMGP (0–100 ng/ml) or transiently transfected with an expression construct containing hMGP (0–200 ng/well). Twenty-four hours after initiation of treatment or transfection, TGF-β1 expression was determined using real-time PCR and normalized to GAPDH expression. No significant change was observed after hMGP treatment, but a mild decrease was seen after hMGP transfection. Expression of bovine and human MGP after transfection of the hMGP construct was comparable to MGP seen after hMGP transfection. Expression of bovine and human MGP after transfection of the hMGP construct was comparable to MGP seen after hMGP transfection.

B, LAP-β1 medium. LAP-β1 expression was determined using real-time PCR and normalized to GAPDH expression. No significant change was observed after hMGP treatment, but a mild decrease was seen after hMGP transfection. Expression of bovine and human MGP after transfection of the hMGP construct was comparable to MGP seen after hMGP transfection.

C, ALK1, ALK5, and GAPDH. ALK1 and ALK5 expression was determined using real-time PCR and normalized to GAPDH expression. No significant change was observed after hMGP treatment, but a mild decrease was seen after hMGP transfection. Expression of bovine and human MGP after transfection of the hMGP construct was comparable to MGP seen after hMGP transfection.

DISCUSSION

MGP expression has been reported in vascular endothelium in species as different as human and teleost fish (1, 2), suggesting that MGP has a role in EC function. Several features in MGP deficiency (3–8) suggest involvement of vascular endothelium, including peripheral pulmonary stenosis, that may result from a failure in angiogenesis or vessel fusion (9, 10), arterial calcification that may relate to endothelial dysfunction during vascular maturation (11), and loss of architecture and hypertrophic chondrocytes in the bone growth plate that may relate to disturbed vascularization (12). Increased MGP expression has been reported in tube-forming EC as determined by subtractive hybridization (13), and in myometrial EC treated with VEGFs as determined by microarray analysis (14). Altered expression of MGP has also been reported in several types of malignancies, including glioma, ovarian cancer, colorectal adenocarcinoma, and urogenital malignancies (15–18), possibly related to vascularization.

In this study we demonstrated for the first time that MGP affects EC function and promotes release of VEGF-A and bFGF. Focusing on VEGF, we found that MGP increased VEGF expression through a previously unrecognized enhancement of TGF-β1 signaling through the ALK1-Smad1/5 pathway. The inhibitory effects on BMP-2 activity are consistent with our previous results demonstrating that MGP is able to inhibit BMP-2 activity in cells of mesenchymal origin, including pluripotent C3H10T1/2 cells (19), marrow stromal cells (20), and calcifying vascular cells (21). BMP-2 belongs to the same superfamily of TGF-β growth factors as TGF-β1 (22–25) and is critical for morphogenesis and bone formation. Together, our results suggest that MGP affects several of the TGF-β growth factors, thereby having the ability to alter the overall response to TGF-β growth factors. This concept would be consistent with the wide range of vascular and non-vascular abnormalities seen in MGP deficiency (3–8), and may also be consistent with a role for combined TGF-β/BMP regulation in SMC differentiation. The Krüppel-like transcription factor 4 (KLF4/GKLF) has been identified as a target of both BMP and TGF-β1 in regulation of vascular SMC phenotype (41), and may be an important mediator. Such a regulatory step would likely occur at a stage subsequent to the specification of arteries and veins, because only arterial calcification is seen in MGP deficiency (3, 4).

TGF-β1 is expressed in the vascular intima during development (42), where it could affect both EC and SMC. Heterozygous knockout mice for TGF-β1 with reduced levels of TGF-β1 in the aorta have an endothelium that is more easily activated by cholesterol-enriched diets, and reduced SMC differentiation (43). TGF-β1 has been shown to be affected by different forms of fluid shear stress in BAEC (44) and may play a role in determining the vessel lumen or caliber size (45). In addition, TGF-β1 is a major modulator of angiogenesis involved in the regulation of both the activation and the resolution phase of angiogenesis (29–31). The TGF-β1/ALK5 pathway has been shown to lead to inhibition of cell migration and proliferation, whereas the TGF-β1/ALK1 pathway induces EC migration and proliferation and can directly antagonize signaling through ALK5 (30, 31). However, EC lacking ALK5 are deficient in TGF-β1/ALK1-induced responses (31), demonstrating interdependence between the two receptors.

BMP-2 is expressed in the embryonic aorta (46), and is known to be involved in interactions between ectodermal and mesodermal cells during development (47, 48). Furthermore, it has a role in EC differentiation; BMP-binding endothelial cell precursor-derived regulator, a recently described BMP antagonist in EC, inhibits both BMP signaling and EC differentiation (49). BMP-2 has been implicated in angiogenesis through osteoblast-derived VEGF-A (50), and BMP-2 stimulated angiogenesis has been reported in developing tumors (51).

Both TGF-β1 and BMP-2 are in locations to potentially interact with MGP during development, because MGP is expressed throughout the developing vascular wall (3). Disturbances in such interactions may yield different results depending on where in the vascular tree they occur, e.g. steno-
ses in the peripheral pulmonary arteries, and calcification in the arteries (3–8).

To define the mechanism by which MGP enhances TGF-β1 activity, we excluded that expression of TGF-β1, ALK1, and ALK5 was increased by MGP. We also excluded that MGP decreased the level of TGF-β1 LAP (37), which might have explained the enhancement.

The effect of MGP on proliferation, migration, and tube formation would be most consistent with an activation of ALK1-Smad1/5 signaling (29), and our immunoblots did show an increase in P-Smad1, indicating that this was the case. In contrast, no increase was detected in P-Smad2/3 indicating that ALK5 signaling was not activated. The TGF-β-responsive luciferase reporter gene did not distinguish between ALK1 and ALK5 activation (31). Furthermore, transfection of constitutively active ALK1 mimicked the effect of MGP on VEGF expression. Together, our results suggest that MGP interferes in receptor-ligand interactions, which is now under further investigation.

There are different ways in which MGP could interfere in receptor-ligand interactions. It may activate TGF-β by direct binding in the extracellular space, analogous to connective-tissue growth factor, another modulator of TGF-β1 and BMP-2 (52). We and other investigators have previously shown pro-
tein-protein interaction between BMP-2 and MGP (20, 53, 54) and modulation of BMP-2 activity (19–21). Alternatively, it may target specific ligand-receptor complexes analogous to egdolgin (55) or betaglycan (56), or it may alter the balance between signaling through the canonical Smad pathway and other, non-canonical signaling pathways (25).

MGP may have a role in lung morphogenesis through TGF-β and VEGF. MGP expression is observed throughout lung morphogenesis. It has been localized to the submucosal layers in the developing respiratory tract in mice (57), and has been associated with lung branching (58, 59). TGF-β and VEGF are both involved in the development and maintenance of lungs (10, 60), where correct spatial and timely expression of TGF-β, TGF-β receptors, and VEGF is crucial for morphogenesis. For instance, constitutively active TGF-β1 perturbs epithelial cell differentiation and formation of pulmonary vasculature (61), and misexpressed VEGF increases pulmonary vasculature but disrupts branching in developing respiratory epithelium (62).

In the growing bone, endothelial cells and pre-osteoblasts invade the calcifying zone of the growth plate, and a balance exists between the osteoblastic differentiation, apoptosis of hypertrophic chondrocytes, and vascularization (12). Low expression of MGP in hypertrophic chondrocytes has been shown to induce apoptosis (63) and may explain the lack of these cells in MGP deficiency. However, in light of our results, MGP deficiency could also explain poor vascular invasion that may result from abnormal TGF-β1 activity and low VEGF expression.

MGP is regarded as an inhibitor of vascular calcification (1, 3–5). However, it is difficult to define the mechanism of this protective role of MGP in atherosclerotic plaques. In addition to endothelial expression, MGP expression has been detected in vascular medial cells and macrophages (1, 64), and as a secreted protein, it may affect neighboring cells. It may modulate BMP-2 and inhibit BMP-2-induced osteogenesis as suggested by previous results (21), or it may promote SMC differentiation or fibrosis through modulation of TGF-β1 activity (65). Alternatively, MGP may affect calcification through plate angiogenesis (12). Altogether, our results support a role for MGP in endothelial cell function and in altering the overall cellular response to TGF-β growth factors.

REFERENCES

1. Engelse, M. A., Neele, J. M., Bronckers, A. L. K. K., Pannekoek, H., and de Vries, C. J. M. (2001) Cardiovasc. Res. 52, 281–289.
2. Simes, D. C., Williamson, M. K., Ortiz-Delgado, J. B., Viegas, C. S. B., Price, P. A., and Cancela, M. L. (2003) J. Bone Miner. Res. 18, 244–259.
3. Luo, G., Ducy, P., McKeever, M. D., Pinero, G. J., Loyer, E., Behringer, R. R., and Karsenty, G. (1995) Nature 386, 78–81.
4. Meier, M., Weng, L. P., Alexandrakis, E., Ruschoff, J., and Goeckenjan, G. (2004) Eur. Respir. J. 17, 566–569.
5. Munroe, P. B., Olgnurturk, R. O., Fynes, J. P., Van Maldenber, L. V., and Azzouz, E. M. (1998) Am. J. Med. Genet. 78, 182–187.
6. Zierieisen, F., De Munter, C., and Perlmutter, N. (1993) Pediatr. Radiol. 23, 314–315.
7. Cornes, E. J., Dawson, M., and Lowry, R. B. (1986) Am. J. Med. Genet. 24, 289–294.
8. deMello, D. E., and Reid, L. M. (2000) Ped. Dev. Pathol. 3, 439–449.
9. Warburton, D., Schwarz, M., Tefft, D., Flores-Delgado, G., Anderson, K. D., and Cardoso, W. V. (2000) Mech. Dev. 35, 52–61.
10. Hirschi, K. K., Skalak, T. C., Peirce, S. M., and Little, C. D. (2002) Ann. N. Y. Acad. Sci. 961, 223–242.
11. Gerber, H.-P., and Ferrara, N. (2000) Trends Cardiovasc. Med. 10, 223–228.
12. Gerber, H.-P., and Ferrara, N. (2000) Trends Cardiovasc. Med. 10, 223–228.
13. Ziereisen, F., De Munter, C., and Perlmutter, N. (1993) Pediatr. Radiol. 23, 314–315.