Journal of Cellular Biochemistry 90:756–765 (2003)

Matrix GLA Protein and BMP-2 Regulate Osteoinduction in Calcifying Vascular Cells

Amina F. Zebboudj,[1] Victoria Shin,[1] and Kristina Boström[1,2]*

[1]Division of Cardiology, David Geffen School of Medicine at UCLA, Los Angeles, California 90095-1679
[2]Molecular Biology Institute, UCLA, Los Angeles, California 90095-1679

Abstract Expression of matrix GLA protein (MGP), an alleged calcification inhibitor, is increased in calcified arteries. We used calcifying vascular cells (CVC) that form calcified nodules in vitro to clarify the importance of MGP in vascular cell calcification and differentiation. Unexpectedly, MGP dose-dependently increased calcification in CVC. It also increased expression of the osteogenic marker Cbfal, while decreasing expression of the smooth muscle marker α-actin as assessed by immunoblotting. Bone morphogenetic protein-2 (BMP-2), a known osteoinductive factor also increased calcification and osteogenic differentiation in CVC. We hypothesized that the effect of MGP was linked to that of BMP-2 since previous studies show that MGP modulates BMP-2 activity. Therefore, we compared the effect of MGP at different levels of exogenous BMP-2. Results showed that high BMP-2 levels significantly increased the stimulatory effect of low levels of MGP. A relative inhibition of calcification was observed at intermediate levels of MGP and a trend towards renewed stimulation at high levels of MGP. Thus, addition of MGP either promoted or inhibited calcification, depending on the relative amounts of BMP-2 and MGP. This was confirmed in human CVC with different relative expression of BMP-2 and MGP. Calcification in CVC with high relative expression of BMP-2 was inhibited by MGP, while calcification in CVC with low relative expression of BMP-2 was stimulated by MGP. MGP and BMP-2 both accelerated nodule formation, but had opposite effects on nodule size; MGP decreased while BMP-2 increased nodule size. The effect of BMP-2 may partly be explained by a BMP-2 induced decrease in MGP expression. Together, our results suggest that the effect of MGP on calcification and osteogenic differentiation is determined by availability of BMP-2. J. Cell. Biochem. 90: 756–765, 2003.

Key words: vascular calcification; matrix GLA protein; bone morphogenetic protein-2; calcifying vascular cells

Matrix GLA protein (MGP) is expressed in embryonic and adult aorta [Shanahan et al., 1994; Luo et al., 1997]. It is an alleged calcification inhibitor since MGP-deficiency in mice [Luo et al., 1997] and humans [Meier et al., 2001] results in severe vascular calcification and alterations in vascular cell differentiation [Luo et al., 1997]. Yet, MGP expression is increased in calcified atherosclerotic plaques and Mönckeberg's disease [Shanahan et al., 1994, 1999; Dhore et al., 2001; Engelse et al., 2001], possibly in attempt to limit calcification.

We have previously shown that MGP affects differentiation induced by bone morphogenetic protein-2 (BMP-2), a potent osteoinductive factor [Wozney and Rosen, 1998], in multipotent mesenchymal cells [Boström et al., 2001] and marrow stromal cells [Zebboudj et al., 2002]. Furthermore, we and others have shown a direct protein–protein interaction between MGP and BMP-2 [Wallin et al., 2000; Zebboudj et al., 2002] that may provide a way for MGP to affect calcification. In the embryo, BMP-2 is critical for cardiovascular development evidenced by early vascular failure in BMP-2 knockout mice [Zhang and Bradley, 1996]. In the adult, however, it is believed to contribute to vascular calcification, since its expression has been

Grant sponsor: NIH; Grant number: HL04270; Grant sponsor: The American Heart Association (National); Grant sponsor: The Howard Hughes Medical Institute; Grant sponsor: The Laubisch Fund.

*Correspondence to: Kristina Boström, MD, PhD, Division of Cardiology, David Geffen School of Medicine at UCLA, Box 951679, Room 47-123 CHS, Los Angeles, CA 90095-1679. E-mail: kbostrom@mednet.ucla.edu

Received 1 August 2003; Accepted 4 August 2003

DOI 10.1002/jcb.10669

detected in calcified, atherosclerotic arteries [Boström et al., 1993; Watson et al., 1994; Dhore et al., 2001], and in calcifying vascular cells (CVC) [Boström et al., 1993; Watson et al., 1994].

CVC is a well-characterized in vitro model for vascular calcification [Boström et al., 1993; Watson et al., 1994; Proudfoot et al., 1998; Tintut et al., 1998]. It is an osteoblast-like subpopulation of bovine or human vascular smooth muscle cells (SMC) that form condensations and nodules, and subsequently undergo osteogenic differentiation and mineralization. In the present study, we used CVC to study the importance of MGP for vascular calcification, and how it relates to BMP-2 activity. Our results showed that changes in MGP levels enhance or inhibit vascular calcification as determined by the availability of BMP-2.

MATERIALS AND METHODS

Cell Culture

Bovine and human CVC were cultured as previously described [Boström et al., 1993; Watson et al., 1994; Tintut et al., 1998]. No supplemental ascorbic acid or β-glycerophosphate was added to the medium. Bovine CVC were used between passages 8 and 20, and human CVC between passages 3 and 10. Bovine CVC were used except where it is indicated that human CVC were used. For experiments, CVC were plated at approximately 80% confluency when endogenous MGP-expression is minimal (see "Results," Fig. 1B). For experiments where CVC were treated with MGP and/or BMP-2, the purified agents or conditioned medium containing MGP and/or BMP-2 were initially added at the time of plating, and the medium was renewed every 3–4 days. Conditioned medium collected from sham-transfected cells was used to equalize the amount of conditioned medium. Bovine MGP was purified and verified by SDS–PAGE and mass spectrometry using previously described methodology [Hale et al., 1991; Loeser et al., 1992]. Purified bovine MGP was solubilized in 6 M guanidine hydrochloride, 0.1 M Tris-HCl, pH 8.0. No more than 0.25 μl was added per ml of culture medium, corresponding to a final concentration of 1.5 mM of guanidine chloride. After addition of MGP, the medium was vortexed extensively before addition to the cells. All experiments were repeated a minimum of 3-times.

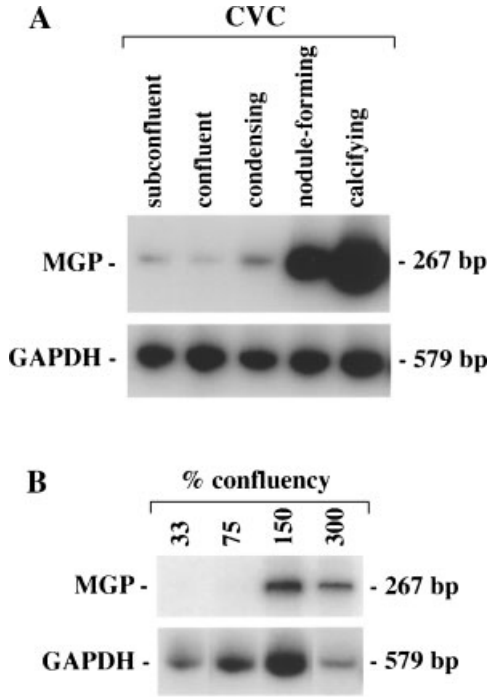

Fig. 1. **A**: Expression of matrix GLA protein (MGP) at different growth stages in bovine calcifying vascular cells (CVC). CVC were plated on day 1, at a density to reach 80% confluency on day 2, when subconfluent cells were harvested. Confluent cells were harvested on day 4, condensing cells on day 7, nodule-forming cells on day 10, and calcifying cells on day 15. Semi-quantitative PCR was performed using specific primers for bovine MGP. GAPDH is shown for comparison. **B**: Expression of MGP in CVC 24 h after plating. CVC were plated at densities adjusted to achieve the indicated percentages of confluency after attachment. Semi-quantitative PCR was performed using specific primers for bovine MGP. GAPDH is shown for comparison. bp, base pairs.

Quantification of BMP-2 and MGP in Conditioned Medium

Conditioned media containing human MGP or human BMP-2 was prepared as previously described [Boström et al., 2001; Zebboudj et al., 2002]. The concentration of BMP-2 in the conditioned media was determined as per manufacturer's instruction using the Quantikine® BMP-2 Immunoassay (R&D Systems, Minneapolis, MN). In previous studies [Boström et al., 2001; Zebboudj et al., 2002], BMP-2 levels were quantified using a bioassay with standards of commercially available BMP-2 (R&D Systems). In comparing the two methods, cell-synthesized BMP-2 was significantly more potent per ng than the commercial BMP-2. Therefore, our previous determinations routinely overestimated the BMP-2 levels by a factor of 5–10. MGP concentrations were previously based on the

bioactivity of cell-synthesized BMP-2 [Boström et al., 2001; Zebboudj et al., 2002], and were, therefore, similarly overestimated.

For direct determination of MGP in conditioned media, we established a new assay. A 16-mer MGP peptide (NRRNANTFISPQQRWR) was diluted to a concentration of 20 μg/ml in coating buffer (0.1 M sodium carbonate, pH 9.6) and used for coating of 96-well plates (50 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 containing 0.02% sodium azide, the plates were ready for use. Samples of MGP-containing conditioned media were tested after two-fold serial dilution with PBS. The assay was standardized using serial dilutions of the peptide used for coating of the wells. In addition, it was standardized using purified bovine MGP (above). The solubilized MGP was diluted in PBS, and vortexed extensively. Four hundred microliter of standard or sample was mixed with 200 μl of anti-MGP (1.5 μg/ml in PBS containing 2% [wt/vol] nonfat dry milk protein) and incubated for 5–10 min at room temperature. The MGP antibody was raised in rabbit against a 32-mer MGP peptide (HESMESYELNPFINRRNANTFISPQQRWRAKV) and kindly provided by Dr. Reidar Wallin (Wake Forest University, NC). Subsequently, each sample was divided into six wells in the coated 96-well plates and incubated for 1 h at 37°C. 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; diluted 1:2,500 in PBS with 2% [wt/vol] nonfat dry milk protein]); 150 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 (pNPP) liquid substrate system (Sigma, St. Louis, MO), 150 μl per well. After 45 min in room temperature the plate was read at 405 nm.

Immunoblotting

Immunoblotting was performed as previously described [Zebboudj et al., 2002]. Blots were incubated with specific antibodies to either smooth muscle (SM)-α-actin (2.7 μg/ml; Bio-Genex, San Ramon, CA), β-actin (1 μg/ml; Sigma), or Cbfal (AML3) (2.5 μg/ml; Oncogene Research Products, San Diego, CA). Densitometry using NIH Image J, version 1.62 (public domain program, internet address http://rsb.info.nih.gov/nih-image) was performed to compare the relative protein levels after normalization to β-actin.

Quantification of Calcium Deposition

Two days prior to quantification of calcium deposition, $CaCl_2$ was added to culture medium to a final concentration of 4 mM. The cellular calcium content was determined as previously described by Jono et al. [2000], and normalized to protein content.

RT-PCR

Semi-quantitative RT-PCR for bovine and human MGP, human BMP-2, and glyceraldehyde 3–phosphate dehydrogenase (GAPDH) was carried out using previously described methods [Boström et al., 2001]. The bovine MGP primers were based on the bovine MGP sequence (GeneBank accession number X07363); BovMGP-5′ (nucleotide 12–25), 5′-AGCCTGCTCCTTCTCTCCATCCTG-3′; BovMGP-3′ (nucleotide 277–254), 5′-CCTGCTTCACCACCTTCTTGATG-3′. The human MGP primers were based on the human MGP sequence (GeneBank accession number X53331); HumMGP-5′ (nucleotide 82–103), 5′-CTTAGCGGTAGTAACTTTGTGT-3′; HumMGP-3′ (nucleotide 232–212), 5′-AGAGCGTTCTCGGATCCTCTC-3′. The BMP-2 primers were based on the human BMP-2 sequence (GeneBank accession number M22489); HumBMP2-5′ (nucleotide 341–364), 5′-CTGTCTTCTAGCGTTGCTGCTTCC-3′; HumBMP-3′ (nucleotide 820–795), 5′-CTGCTATTGTTTCCTAAAGCATCTTG-3′. The GAPDH primers have been described previously [Boström et al., 2001]. The annealing temperatures were 58°C for MGP and GAPDH, and 62°C for BMP-2. Densitometry was performed as per above; GAPDH was used for normalization.

Statistical Analysis

Data was analyzed for statistical significance by ANOVA with post-hoc Scheffe's analysis, unless otherwise stated. The analyses were performed using StatView, version 4.51 (Abacus Concepts, Berkeley, CA). All experiments presented in this study were repeated a minimum of 3-times.

RESULTS

MGP Expression Correlates With Differentiation and Cell Density in CVC

To determine the levels of endogenous MGP expression in the bovine CVC used in this study, we used semi-quantitative RT-PCR analysis since media concentrations may not reflect the correct MGP production due to progressive incorporation of MGP into extracellular matrix [Zebboudj et al., 2002]. Semi-quantitative RT-PCR analysis was performed at different stages of CVC differentiation. Results showed that MGP expression increased as CVC formed nodules and calcified (day 7–15, Fig. 1A), suggesting that MGP plays a role in nodule formation and/or mineralization and osteogenic differentiation. Since MGP is an alleged calcification inhibitor [Luo et al., 1997; Price et al., 2000], we hypothesized that MGP would inhibit CVC calcification.

MGP expression was also determined at different cell densities, since a correlation of MGP expression with cell density has been observed in other cells [Cancela et al., 1997]. Cells were seeded to a final density between 30% (subconfluency) and 300% (superconfluency) after cell attachment. MGP expression was determined 1 day after plating, before nodule formation. It was minimal in subconfluent cells but significantly increased in superconfluent cells (Fig. 2B). As noted in "Materials and Methods," CVC were plated at subconfluency for all experiments.

MGP Dose-Dependently Enhances CVC Calcification

To determine the effect of MGP on bovine CVC differentiation and calcification, CVC were treated for 6 days with MGP in the form of conditioned medium, at concentrations between 0 ng/ml and 80 ng/ml. MGP was first added to the CVC cultures at the time of plating, and after 6 days, calcification and expression of the osteogenic lineage marker Cbfal were measured. In addition, the SMC marker SM-α-actin was used to determine the effect of MGP on SMC differentiation. Results showed that MGP dose-dependently stimulated calcification and osteogenic differentiation as assessed by Cbfal expression. There was a mild increase in calcification at low levels (0.6–4.0 ng/ml) of MGP, and a significant increase at high levels of MGP (>12 ng/ml, Fig. 2A). Note that a non-linear scale is used, to better illustrate changes occurring at low levels of MGP. Cbfal-expression increased in parallel with calcification (Fig. 2B,C). SM-α-actin decreased as more MGP was added.

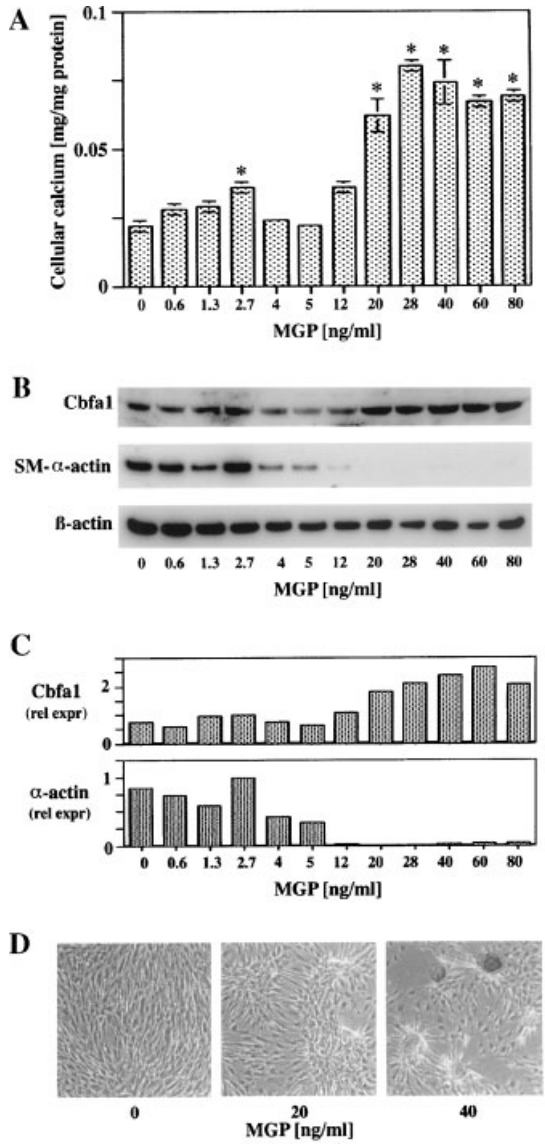

Fig. 2. MGP dose-dependently promotes calcification, osteogenic differentiation, and nodule formation in bovine CVC. **A**: MGP (0–80 ng/ml) was added to CVC at the time of plating, and CVC were treated for a total of 6 days, after which cellular calcium was determined. The results are presented as mean ± SD for triplicate samples. * Indicates statistically significant difference compared to no MGP ($P < 0.001$, Scheffe's test). A non-linear scale is used to better illustrate changes at low MGP concentrations. **B**: Immunoblotting was performed after 6 days of treatment for lineage markers Cbfal (osteogenic), and SM-α-actin (smooth muscle cell (SMC)). β-Actin is shown for comparison. **C**: Relative expression of Cbfal and SM-a-actin, normalized to β-actin. **D**: Morphology of CVC treated with MGP (0, 20, and 40 ng/ml) for 2 days. Original magnification 40×.

β-Actin was used as a loading control. In addition, MGP accelerated nodule formation, and as the MGP level increased, the nodules became progressively smaller and more sharply defined (Fig. 2D). Compared to the spontaneous nodule formation in these CVC, which takes approximately 7–12 days (Fig. 1A), nodule formation was complete within 2–3 days. High alkaline phosphatase activity was observed in the nodules as assessed by staining, as previously described [Watson et al., 1994; Tintut et al., 1998] (data not shown).

Similar effects on calcification and nodule formation were seen when purified bovine MGP was used (data not shown). Due to well-known difficulties solubilizing purified MGP [Hale et al., 1991; Loeser et al., 1992], the final concentration of active MGP varied depending on the success of the solubilization in culture medium. Frequently, precipitated material was observed in the medium after MGP addition, correlating with less activity.

BMP-2 Enhances CVC Calcfication

We and others have previously shown that MGP binds and modulates BMP-2 [Wallin et al., 2000; Boström et al., 2001; Zebboudj et al., 2002], and we hypothesized that the effect of MGP may be linked to that of BMP-2. CVC are known to express BMP-2 [Boström et al., 1993; Watson et al., 1994], which has been presumed to be osteogenic in these cells. However, this has never been tested. To determine the effect of exogenous BMP-2 on differentiation and calcification, bovine CVC were treated with BMP-2 at concentrations between 0 ng/ml and 40 ng/ml. BMP-2 was first added to the CVC cultures at the time of plating, and after 6 days, calcification and expression of Cbfal and SM-α-actin were measured. Results showed a dose-dependent increase in calcification (Fig. 3A) and Cbfal expression, paralleled by a decrease in SM-α-actin expression (Fig. 3B,C). Furthermore, nodule formation was accelerated by BMP-2, however, in contrast to MGP, BMP-2 resulted in progressively larger and less defined nodules (Fig. 3D). Again, nodule formation was complete within 2–3 days, and high alkaline phosphatase activity was observed in the nodules (data not shown).

MGP Effect is Dependent on BMP-2 Levels

The effect of MGP on CVC may result from modulation of the endogenous BMP-2. To determine if higher levels of BMP-2 would alter the effect of MGP, bovine CVC were treated with two concentrations of BMP-2 (6 and 10 ng/ml) in combination with the same range of MGP concentrations as used above. After 6 days,

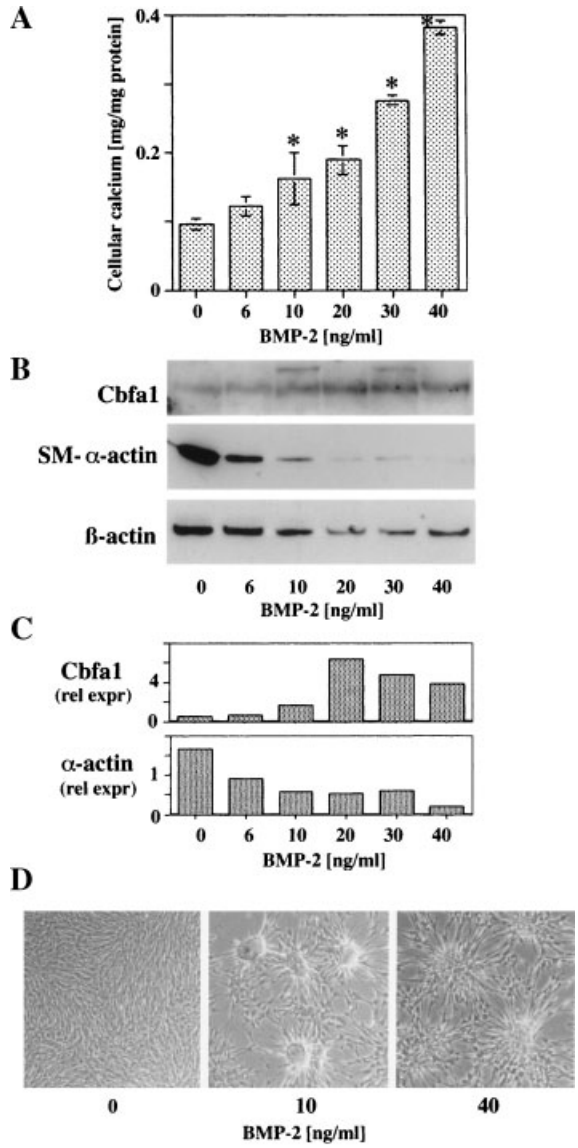

Fig. 3. Bone morphogenetic protein-2 (BMP-2) promotes calcification, osteogenic differentiation, and nodule formation in bovine CVC. **A**: BMP-2 (0–40 ng/ml) was added to CVC at the time of plating, and CVC were treated for a total of 6 days, after which cellular calcium was determined. The results are presented as mean ± SD for triplicate samples. * Indicates statistically significant difference compared to no BMP-2 ($P < 0.001$, Scheffe's test). **B**: Immunoblotting was performed after 6 days of treatment for lineage markers Cbfal (osteogenic), and SM-α-actin (SMC). β-Actin is shown for comparison. **C**: Relative expression of Cbfa1 and SM-a-actin, normalized to β-actin. **D**: Morphology of CVC treated with BMP-2 (0, 10, and 40 ng/ml) for 2 days. Original magnification 40×.

calcification was quantified and the results compared with CVC treated with MGP only (compare Fig. 2A).

The baseline calcification for higher level of BMP-2 increased as shown in Figure 3. In Figure 4, however, baseline calcification is in each case set to zero to better illustrate the effect of MGP. In presence of both 6 and 10 ng/ml of BMP-2, the stimulatory effect of low levels of MGP (0.6–4.0 ng/ml), was significantly enhanced compared to that of MGP alone. At intermediate levels of MGP (9–20 ng/ml), the stimulatory effect decreased; a relative inhibition was seen when compared to the strong stimulation by low MGP levels. At high MGP levels (>20 ng/ml), the stimulatory effect did not increase as dramatically in the presence of BMP-2 as it did for MGP alone. This was especially noticeable for 10 ng/ml of BMP-2. It is possible that addition of more MGP would result in further stimulation, however, this could not be determined due to experimental limitations. Thus, at each combination of BMP-2 and MGP, addition of MGP either increased or decreased calcification, depending on the relative amounts of BMP-2 and MGP.

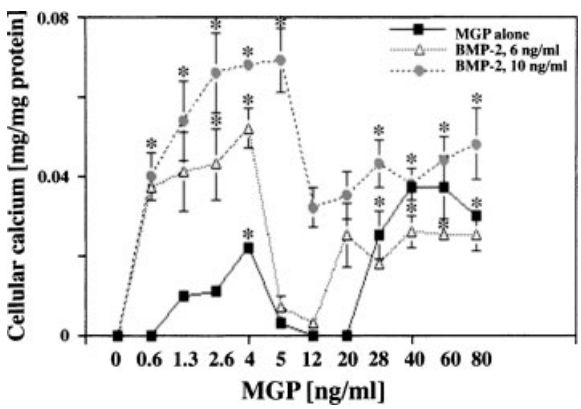

Fig. 4. Effect of increasing MGP concentrations at different BMP-2 concentrations on calcification in bovine CVC. MGP (0–80 ng/ml), without BMP-2, or with 6 or 10 ng/ml of BMP-2 was added to CVC at the time of plating, and CVC were treated for a total of 6 days. Cellular calcium was determined and the results are presented as mean ± SD for triplicate samples. The baseline calcification for each BMP-2 concentration increased as shown in Figure 3, however, baseline calcification is here set to zero at 0 ng/ml of MGP for all BMP-2 concentrations. The values represent the increases in cellular calcium above baseline. * Indicates statistically significant difference compared to no MGP ($P < 0.001$, Scheffe's test). A non-linear scale is used to better illustrate changes at low MGP concentrations.

Differential Effects of MGP on Rapidly and Slowly Mineralizing Human CVC

Based on the above findings, we predicted that MGP would have an inhibitory effect on calcification in CVC with high expression of BMP-2 relative to that of MGP. It would mimic the relative inhibition seen when MGP levels increased from low to intermediate in the presence of 6 or 10 ng/ml of BMP-2 (Fig. 4). Furthermore, we predicted that MGP would have a stimulatory effect on calcification in CVC with low expression of BMP-2 relative to MGP. It would mimic the curve for MGP alone (Fig. 4).

We used two preparations of human CVC, which have similar characteristics to bovine CVC [Boström et al., 1993; Proudfoot et al., 1998]. The first preparation had an increased rate of nodule formation and calcification; calcified nodules formed after only 5–7 days. The second preparation was more similar to the bovine CVC, but required approximately 21 days to form calcified nodules. Both preparations expressed similar amounts of BMP-2 at 80–90% confluency, as assessed by semi-quantitative RT-PCR (Fig. 5). However, the slow CVC expressed approximately 2.2-times more MGP than the rapid CVC, thereby decreasing the relative expression of BMP-2.

Both CVC preparations were treated with MGP (0–80 ng/ml), and calcification was determined after 6 days. As predicted, calcification was inhibited by high levels of MGP in the rapid CVC (Fig. 6A). Also as predicted, calcification was enhanced by high levels of MGP in the slow

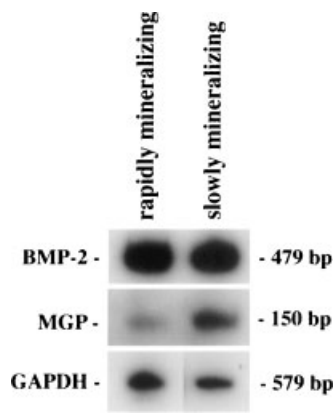

Fig. 5. BMP-2 and MGP expression in rapidly and slowly mineralizing human CVC. Human CVC were harvested on day 5, and semi-quantitative PCR was performed using specific primers for Human BMP-2 and MGP. GAPDH is shown for comparison. bp, base pairs.

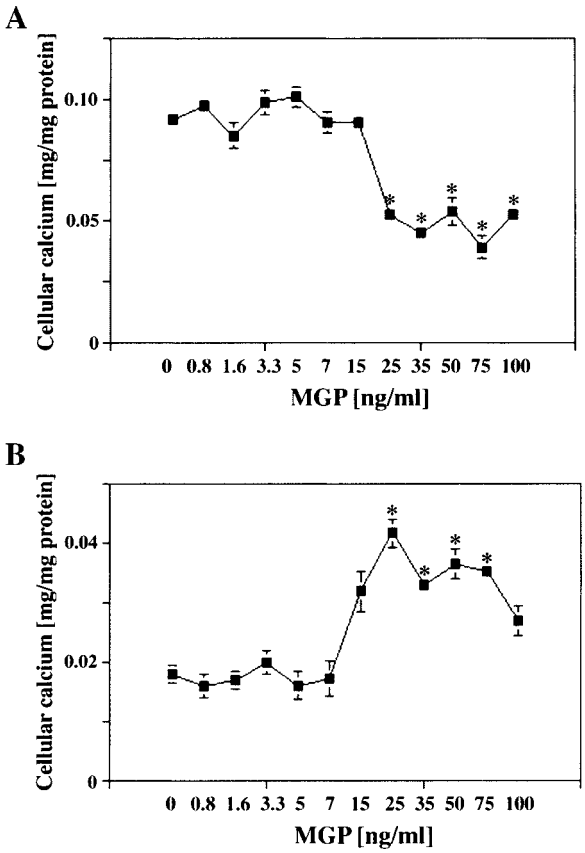

Fig. 6. Effect of increasing MGP concentrations in rapidly and slowly mineralizing human CVC. MGP (0–100 ng/ml) was added to rapidly and slowly mineralizing human CVC at the time of plating, and CVC were treated for a total of 6 days. Cellular calcium was determined and the results are presented as mean ± SD for triplicate samples. * Indicates statistically significant difference compared to no MGP ($P < 0.001$, Scheffe's test). A non-linear scale is used to better illustrate changes at low MGP concentrations. **A**: Rapidly mineralizing human CVC with high BMP-2 expression relative to MGP, decrease calcification in response to increased MGP-levels. **B**: Slowly mineralizing human CVC with low BMP-2 expression relative to MGP, increase calcification in response to increased MGP-levels.

CVC (Fig. 6B). Thus, addition of MGP conditionally promotes or inhibits calcification, depending on the relative amounts of BMP-2 and MGP already present.

BMP-2 Decrease MGP Expression

The progressively larger and less defined nodules observed in BMP-2 treated CVC (Fig. 3D) suggested that BMP-2 decrease MGP expression. To determine whether BMP-2 decreased MGP expression, CVC were treated with BMP-2 at concentration between 0 ng/ml and 40 ng/ml. BMP-2 was added to the CVC cultures at the time of plating, and after 4 days, levels of MGP expression was determined using semi-quantitative RT-PCR analysis. Results showed a dose-dependent decrease in MGP expression when normalized to GAPDH expression. MGP levels decreased to approximately 40% of levels in non-treated cells (Fig. 7).

DISCUSSION

Increased MGP expression in atherosclerotic plaques was first reported by Shanahan et al. [1994], and it was hypothesized that it may predispose to tissue calcification. In addition, it was reported that MGP expression in cartilage was highest during the calcification period [Barone et al., 1991], possibly promoting calcification. However, this view was changed by the findings in MGP knockout mice [Luo et al., 1997], where severe calcification of arteries and normally non-calcifying cartilage was observed. It was associated with profound alterations in differentiation of arterial SMC, which were replaced by chondrocyte-like cells undergoing endochondral bone formation. Similarly, Price et al. [2000] were able to induce vascular calcification in young, actively growing rats by using warfarin, an inhibitor of the γ-carboxylation necessary for functional MGP. However, no change in cell differentiation was observed, suggesting there may be differences in MGP

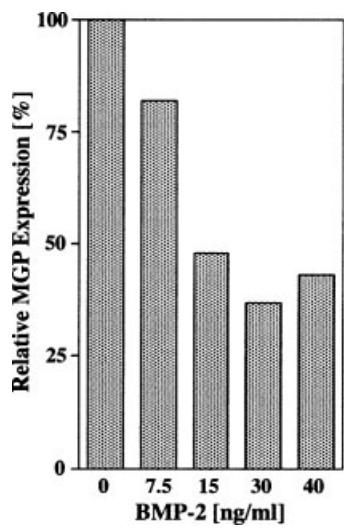

Fig. 7. Effect of increasing BMP-2 concentration on MGP expression in bovine CVC. BMP-2 (0–40 ng/ml) was added to CVC at the time of plating, and CVC were treated for a total of 4 days. Semi-quantitative RT-PCR was performed using specific primers for bovine MGP. Relative expression of MGP is shown as the percentage of MGP expression in CVC treated with 0 ng/ml of BMP-2, normalized to GAPDH. A representative experiment out of four independent experiments is shown.

function depending on the developmental stage. Together, the findings led to the speculation that increased MGP expression is an attempt to limit calcification in diseased arteries.

Shortly after publication of the MGP knockout mouse, the genetic defect in the human Keutel syndrome was located to the *MGP* gene [Munroe et al., 1999]. Patients with Keutel syndrome have similar vascular calcification [Meier et al., 2001], but in addition multiple morphogenic defects including peripheral pulmonary stenosis, midfacial hypoplasia, and brachytelephalangism [Teebi et al., 1998; Munroe et al., 1999; Meier et al., 2001]. These are mirrored in the short snout and legs in the MGP knockout mouse [Luo et al., 1997]. During embryogenesis, MGP has been reported to be predominantly expressed at epithelial–mesenchymal interfaces [Luo et al., 1995], mainly of lung and limb buds, which may contribute to organ morphogenesis.

The BMPs belong to the TGFβ superfamily of growth factors, and have crucial roles in bone and cartilage development and in morphogenesis [Chang et al., 2002]. We and others have previously shown that MGP binds and modulates BMP-2 activity [Wallin et al., 2000; Zebboudj et al., 2002]. BMP-2 is expressed in embryonic vasculature and is critical for development [Zhang and Bradley, 1996]. It is also a potent osteoinductive factor, and may promote calcification of atherosclerotic plaques [Boström et al., 1993; Watson et al., 1994; Dhore et al., 2001], which is supported by our results; both osteogenic differentiation and calcification in CVC were enhanced by BMP-2.

Our results suggest that MGP is a conditional enhancer or inhibitor of BMP-2 induced calcification, and that enhancement or inhibition depends on the specific BMP-2 level relative to that of MGP at the time when MGP is increased. In CVC, which express BMP-2 [Boström et al., 1993; Watson et al., 1994], the effect of MGP alone is likely a modulation of the endogenous BMP-2. Increased BMP-2 levels altered the effect of an identical concentration of MGP. The differential effect of MGP was confirmed in human CVC with different BMP-2 expression relative to that of MGP. Addition of MGP decreased calcification where relative BMP-2 levels were high, and increased calcification where relative BMP-2 levels were low. In part, the dependence on BMP-2 for MGP to enhance or inhibit osteoinduction may result from the ability of BMP-2 to decrease MGP expression. Thus, the differential effect of MGP on osteoinduction may be a combined effect of MGP-binding BMP-2 and BMP-2 decreasing MGP expression. The effect of MGP in CVC is reminiscent to the differential effect of MGP on alkaline phosphatase activity in M2-10B4 cells treated with BMP-2 [Zebboudj et al., 2002].

Differential effects of MGP has also been observed in chondrocytes. MGP has a specific expression profile in developing chondrocytes undergoing endochondral ossification [Luo et al., 1995]. It is expressed in resting, proliferative, and late hypertrophic chondrocytes, but absent from early hypertrophic chondrocytes, and from osteoblasts. Yagami et al. [1999] showed that the effect of warfarin on mineralization in chondrocyte cultures was highly selective. Warfarin triggered mineralization in hypertrophic but not immature chondrocyte cultures. This is consistent with results from Newman et al. [2001], who showed that overexpression of MGP in hypertrophic chondrocytes does reduce mineralization.

We speculate that dose-dependent regulation of BMP-2 is a mechanism by which MGP may affect morphogenesis. BMP and BMP-inhibitors have been shown to participate in morphogenetic gradients, a concept well established in developmental biology that allows for differential effects of a so called morphogen [Gurdon and Bourillot, 2001; Teleman et al., 2001; Eldar et al., 2002]. It has also been reported that OP-1 (BMP-7), closely related to BMP-2, have both stimulatory and inhibitory effects on renal branching morphogenesis depending on the dose [Piscione et al., 1997]. Interestingly, Engelse et al. [2001] reported the presence of a gradient of MGP from the luminal to the adventitial side of atherosclerotic arteries, which could result in differential effects on BMP-2 activity.

Formation of CVC condensations and nodules was affected by BMP-2 and MGP. Both proteins accelerated nodule formation in CVC, though BMP-2 increased and MGP decreased nodule size. However, the inhibitory effect of BMP-2 on MGP expression, suggest that the effect of BMP-2 on nodules occurs by decreasing MGP. The effect of BMP-2 may be comparable to that seen with BMP-2 overexpression in mesenchyme surrounding early cartilage condensations in the developing chick limb [Duprez et al., 1996]. An increase in the volume of cartilage

elements was observed, without increased proliferation of chondrocytes, suggesting increased recruitment of precursors. The effect of MGP was consistent with the observation by Canfield et al. [2000], that anti-MGP antibodies slowed nodule formation in pericytes, which are similar to CVC [Boström et al., 1993]. It is possible that cell organization is the primary effect of MGP and BMP-2 treatment in CVC while the effects on mineralization and Cbfal expression may be mediated by other factors. Results from Tintut et al. [1998] and Parhami et al. [2002] suggest that nodule formation is a separate process from calcification. cAMP treatment of CVC abolished nodule formation while enhancing calcification [Tintut et al., 1998], and inhibition of calcification by HDL did not affect nodule formation [Parhami et al., 2002]. Alternatively, MGP and BMP-2 may act in sequence at different steps in cell organization and cell differentiation to contribute to the formation of skeletal elements.

ACKNOWLEDGMENTS

We thank Dr. Reidar Wallin, Wake Forest University, NC, for antibodies to MGP.

REFERENCES

Barone LM, Owen TA, Tassinari MS, Bortell R, Stein GS, Lian JB. 1991. Developmental expression and hormonal regulation of the rat matrix Gla protein (*MGP*) gene in chondrogenesis and osteogenesis. J Cell Biochem 46: 351–365.

Boström K, Watson KE, Horn S, Wortham C, Herman IM, Demer LL. 1993. Bone morphogenetic protein-2A expression in human atherosclerotic lesions. J Clin Invest 91: 1800–1809.

Boström K, Tsao D, Shen S, Wang Y, Demer LL. 2001. Matrix GLA protein modulates differentiation induced by bone morphogenetic protein-2 in C3H10T1/2 cells. J Biol Chem 276:14044–14052.

Cancela ML, Hu B, Price PA. 1997. Effect of cell density and growth factors on matrix GLA protein expression by normal rat kidney cells. J Cell Physiol 171:125–134.

Canfield AE, Doherty MJ, Kelly V, Newman B, Farrington C, Grant ME, Boot-Handford RP. 2000. Matrix Gla protein is differentially expressed during the deposition of a calcified matrix by vascular pericytes. FEBS Letters 487:267–271.

Chang H, Brown CW, Matzuk MM. 2002. Genetic analysis of the mammalian transforming growth factor-β superfamily. Endocrine Rev 23:787–823.

Dhore CR, Cleutjens JPM, Lutgens E, Cleutjens KBJM, Geusens PPM, Kitslaar PJEHM, Tordoir JHM, Spronk HMH, Vermeer C, Daemen MJAP. 2001. Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques. Arterioscler Thromb Vasc Biol 21:1998–2003.

Duprez D, Bell EJ, Richardson MK, Arcger CW, Wolpert L, Brickell PM, Francis-West PH. 1996. Overexpression of BMP-2 and BMP-4 alter the size and shape of developing skeletal elements in the chick limb. Mech Dev 57:145–157.

Eldar A, Dorfman R, Weiss D, Ashe H, Shilo BZ, Barkai N. 2002. Robustness of the BMP morphogen gradient in *Drosophila* embryonic patterning. Nature 419:261–262.

Engelse MA, Neele JM, Bronckers ALJJ, Pannekoek H, de Vries CJM. 2001. Vascular calcification: Expression patterns of the osteoblast-specific gene core binding factor α-1 and the protective factor matrix gla protein in human atherogenesis. Cardiovasc Res 52:281–289.

Gurdon JB, Bourillot P-Y. 2001. Morphogen gradient interpretation. Nature 413:797–803.

Hale JE, Williamson MK, Price PA. 1991. Carboxyl-terminal proteolytic processing of matrix Gla protein. J Biol Chem 266:21145–21149.

Jono S, Peinado C, Giachelli CM. 2000. Phosphorylation os osteopontin is required for inhibition of vascular smooth muscle cell calcification. J Biol Chem 275:20197–20203.

Loeser R, Carlson CS, Tulli H, Jerome WG, Miller L, Wallin R. 1992. Articular-cartilage matrix γ-carboxyglutamic acid-containing protein. Biochem J 282:1–6.

Luo G, D'Souza R, Hogue D, Karsenty G. 1995. The matrix Gla protein gene is a marker of the chondrogenesis cell lineage during mouse development. J Bone Miner Res 10:325–334.

Luo G, Ducy P, McKee MD, Pinero GJ, Loyer E, Behringer RR, Karsenty G. 1997. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. Nature 386:78–81.

Meier M, Weng LP, Alexandrakis E, Ruschoff J, Goeckenjan G. 2001. Tracheobronchial stenosis in Keutel syndrome. Eur Respir J 17:566–569.

Munroe PB, Olgunturk RO, Fryns J-P, Van Maldergem L, Ziereisen F, Yuksel B, Gardiner RM, Chung E. 1999. Mutations in the gene encoding the human matrix Gla protein cause Keutel syndrome. Nat Genet 21:142–144.

Newman B, Gigout LI, Sudre L, Grant ME, Wallis GA. 2001. Coordinated expression of matrix Gla protein is required during endochondral ossification for chondrocyte survival. J Cell Biol 154:659–666.

Parhami F, Basseri B, Hwang J, Tintut Y, Demer LL. 2002. High-density lipoprotein regulates calcification of vascular cells. Circ Res 91:570–576.

Piscione TD, Yager TD, Gupta IR, Grinfeld B, Pei Y, Attisano L, Wrana JL, Rosenblu ND. 1997. BMP-2 and OP-1 exert direct and opposite effects on renal branching morphogenesis. Am J Physiol 273:F961–F975.

Price PA, Faus SA, Williamson MK. 2000. Warfarin-induced artery calcification is accelerated by growth and vitamin D. Arterioscler Thromb Vasc Biol 20:317–327.

Proudfoot D, Skepper JN, Shanahan CM, Weissberg PL. 1998. Calcification of human vascular cells in vitro is correlated with high levels of matrix Gla protein and low levels of osteopontin expression. Arterioscler Thromb Vasc Biol 18:379–388.

Shanahan CM, Cary NR, Metalfe JC, Weissberg PL. 1994. High expression of genes for calcification-regulating proteins in human atherosclerotic plaques. J Clin Invest 93:2393–2402.

Shanahan CM, Cary NRB, Salisbury JR, Proudfoot D, Weissberg PL, Edmonds ME. 1999. Medial localization of

mineralization-regulating proteins in association with Mönckeberg's sclerosis. Circulation 100:2168–2176.

Teebi AS, Lambert DM, Kaye GM, Al-Fifi S, Tewfik TL, Azouz EM. 1998. Keutel syndrome: Further characterization and review. Am J Med Genet 78:182–187.

Teleman AA, Strigini M, Cohen S. 2001. Shaping morphogen gradients. Cell 105:559–562.

Tintut Y, Parhami F, Boström K, Jackson SM, Demer LL. 1998. cAMP stimulates osteoblast-like differentiation of calcifying vascular cells. J Biol Chem 273:7547–7553.

Wallin R, Cain D, Hutson SM, Sane DC, Loeser R. 2000. Modulation of the binding of matrix Gla protein (MGP) to bone morphogenetic protein-2 (BMP-2). Thromb Haemost 84:1039–1044.

Watson KE, Boström K, Ravindranath R, Lam T, Norton B, Demer LL. 1994. TGF-β1 and 25-hydroxycholesterol stimulate osteoblast-like vascular cells to calcify. J Clin Invest 93:2106–2113.

Wozney JM, Rosen V. 1998. Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair. Clin Orthop 346:26–37.

Yagami K, Suh J-Y, Enomoto-Iwamoto M, Koyama E, Abrams WR, Shapiro IM, Pacifici M, Iwamoto M. 1999. Matrix GLA protein is a developmental regulator of chondrocyte mineralization and, when constitutively expressed, blocks endochondral and intramembraneous ossification in the limb. J Cell Biol 147:1097–1108.

Zebboudj AF, Imura M, Boström K. 2002. Matrix GLA protein, a regulatory protein for bone morphogeneticprotein-2. J Biol Chem 277:4388–4394.

Zhang H, Bradley A. 1996. Mice deficient for BMP-2 are nonviable and have defects in amnion/chorion and cardiac development. Development 122:2977–2986.