Expression of matrix Gla protein (MGP) in an in vitro model of vascular calcification

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Abstract To investigate the role of matrix Gla protein (MGP), which can bind mineral ions through γ-carboxylated glutamic acid residues, in vascular calcification, we examined the expression of MGP in an in vitro calcification model by using bovine vascular smooth muscle cells (BVSMC). The expression of MGP mRNA was decreased during BVSMC calcification and its levels were inversely correlated with the quantities of BVSMC calcification. MGP mRNA expression was restored to the level of uncalcified control by inhibiting BVSMC calcification with bisphosphonates. These data suggest that the expression of MGP gene is modulated in the development of vascular calcification.

Key words: Matrix Gla protein; β-Glycerophosphate; Vascular calcification; Atherosclerosis; Vascular smooth muscle cell; Bisphosphonate

1. Introduction

Matrix Gla protein (MGP), which was originally isolated from bovine bone, belongs to a family of extracellular mineral-binding proteins called Gla proteins [1–3]. It has been reported subsequently that the MGP gene is expressed in various organs including lung, kidney, heart, and cartilage [4,5]. Although Gla proteins bind mineral and mineral ions through γ-carboxylated glutamic acid residues [2], the functional roles of MGP even in skeletal tissues remain obscure. However, it has been recently reported that MGP-deficient mice develop diffuse calcification of arteries and cartilage [6]. Therefore, it is likely that MGP functions as an inhibitor of calcification in noncalcified tissues such as arteries and cartilage.

Although vascular calcification was generally regarded as an unregulated and end-stage process of atherosclerosis, it has been becoming recognized that it is an active and regulated process similar to osteogenesis [7]. In calcified atherosclerotic plaques, the expressions of bone matrix proteins, including osteopontin and MGP were detected by immunohistochemical technique and in situ hybridization [8]. In order to clarify the molecular mechanism of vascular calcification, we developed an in vitro calcification system in which diffuse calcification can be induced by culturing bovine vascular smooth muscle cells (BVSMC) in the presence of β-glycerophosphate (β-GP) [9]. To investigate the role of MGP in vascular calcification, we examined the expression of MGP by using this in vitro calcification model. In this study, we demonstrated that expression of MGP mRNA is modulated in the development of BVSMC calcification.

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Abbreviations: MGP, matrix Gla protein; BVSMC, bovine vascular smooth muscle cells; β-GP, β-glycerophosphate; EHDP, etidronate

2. Materials and methods

2.1. Reagents

Media, fetal bovine serum (FBS), and sodium pyruvate were purchased from Gibco (Grand Island, NY). β-Glycerophosphate was obtained from Sigma (St. Louis, MO). Etidronate (EHDP) and alendronate were kindly provided from Sumitomo Pharmaceuticals (Osaka, Japan) and Merck Sharp and Dohme Research Laboratories (West Point, PA), respectively. Unless otherwise mentioned, all other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Cell culture and in vitro calcification

BVSMC were acquired by an explant method as previously described [9]. Briefly, the tunica media was isolated from bovine aorta. The tissue was fragmented (1 to 2 mm^3) and placed in a 10-cm culture dish and cultured for several weeks in DMEM containing 4.5 g/l of glucose supplemented with 15% FBS and 10 mM sodium pyruvate at 37°C in a humidified atmosphere containing 5% CO_2. Cells that had migrated from the explants were collected and maintained in the growing medium. The cells up to passage 8 were used for experiments. BVSMC calcification was induced as previously described [9]. Briefly, BVSMC were cultured in the growing medium. After confluence, the cells were seeded in DMEM containing 15% FBS and 10 mM sodium pyruvate in the presence of 10 mM β-glycerophosphate. The medium was replaced with fresh medium every 2 days. In the time-course experiments, the beginning day of culture in calcification medium was defined as day 0.

2.3. Quantification of calcium deposition

The cells were decalcified with 0.6 N HCl for 24 h. The calcium content of HCl supernatant was determined colorimetrically by *o*-cresolphthalein complexone method (Calcium C-test Wako; Wako Pure Chemical Industries, Osaka, Japan) [10]. After decalcification, the cells were washed three times with phosphate-buffered saline (PBS) and solubilized with 0.1 N NaOH/0.1% sodium dodecyl sulfate (SDS). The protein content was measured with a BCA protein assay kit (Pierce, Rockford, IL). The calcium content of cell layer was normalized by protein content.

2.4. Preparation of cDNA probes

Bovine MGP cDNA probe containing a 232-base pair fragment (corresponding to 54 to 285 in the coding region) was obtained by reverse transcription of an mRNA from BVSMC, followed by polymerase chain reaction and subcloning into TA cloning vector (Invitrogen, Carlsbad, CA). Sequences of the obtained cDNA were confirmed by dideoxy sequencing method.

2.5. RNA isolation and Northern blot analysis condition

Total RNA was isolated from BVSMC by extraction with acid guanidium thiocyanate-phenol-chloroform. Twenty micrograms of total RNA were electrophoresed on 1% agarose gels containing formaldehyde and transferred to a nylon filter (Hybond N, Amersham, Buckinghamshire, UK). Blots were prehybridized at 37°C for 24 h in a buffer containing 50% formamide, 3×SSC (1×SSC, 0.15 M NaCl and 15 mM sodium citrate, pH 7.4), 50 mmol/l Tris-HCl (pH 7.5), 0.1% SDS, 20 μg/ml denatured salmon sperm DNA, and 1×Denhardt's solution and then hybridized at 37°C for

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48 h with cDNA probe for bovine MGP that was labeled with [α-^{32}P]dCTP (3000 Ci/ml; New England Nuclear, Boston, MA) by use of a random priming method (Megaprime cDNA labeling system, Amersham, Buckinghamshire, England). Blots were washed and autoradiographed with X-ray film at −70°C. The amounts of RNA were quantified by densitometric scanning and normalized by comparison with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [11].

3. Results

As previously described, 10 mM β-GP increased calcium deposition in a time-dependent manner (Fig. 1a). We examined the effect of calcification on MGP mRNA expression by Northern blot analysis. As shown in Fig. 1b, MGP mRNA expression was depressed in the development of BVSMC calcification in a time-dependent manner and on day 6

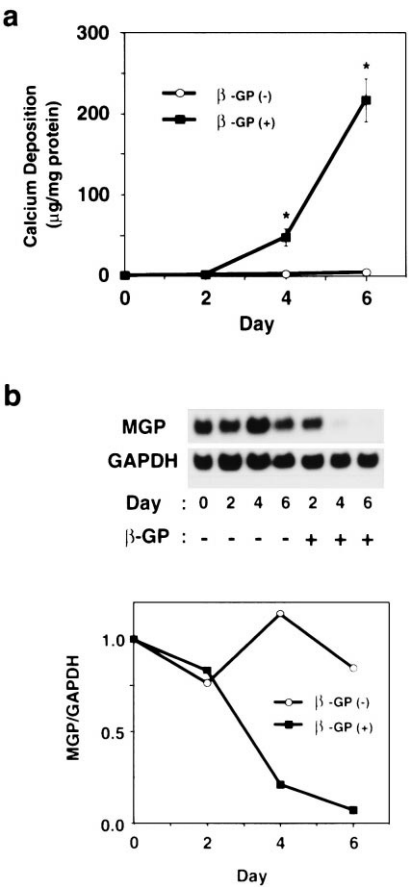

Fig. 1. Time courses of BVSMC calcification (a) and expression of MGP mRNA (b). The cells were cultured as described in Section 2. β-GP (+) and (−) indicate the presence and absence of β-glycerophosphate, respectively. Calcified group is indicated by closed square and uncalcified control is indicated by open circle. a: The calcium content was measured at the indicated times by *o*-cresolphthalein complexone method, normalized by cellular protein content, and is presented as mean ± S.E.M. The differences compared with uncalcified control at each time point were statistically significant ($*P < 0.05$, Fisher's PLSD). b: Twenty micrograms of total RNA from BVSMC at the indicated times were electrophoresed, blotted, and probed with cDNA of bovine MGP. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Upper panel, autoradiograph of Northern analysis of MGP. Lower panel, densitometric analysis of the autoradiograms was performed and the results are presented as the ratio of MGP to GAPDH.

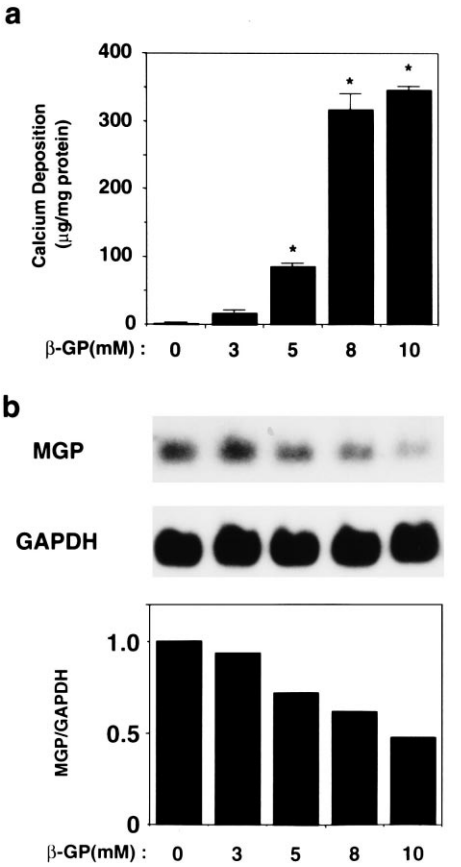

Fig. 2. Dose-dependent effects of β-GP on BVSMC calcification (a) and expression of MGP mRNA (b). The cells were cultured for 4 days as described in Section 2. a: The calcium content was measured by *o*-cresolphthalein complexone method, normalized by cellular protein content, and is presented as mean ± S.E.M. The differences compared with uncalcified control were statistically significant ($*P < 0.05$, Fisher's PLSD). b: Twenty micrograms of total RNA from BVSMC were electrophoresed, blotted, and probed with cDNA of bovine MGP. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Upper panel, autoradiograph of Northern analysis of MGP. Lower panel, densitometric analysis of the autoradiograms was performed and the results are presented as the ratio of MGP to GAPDH.

the level of MGP mRNA was reached to 8.3% of that of uncalcified control. We next examined the relationship between the contents of calcium deposition and the levels of MGP mRNA. Calcium deposition was dose-dependently increased by the addition of β-GP (Fig. 2a). On the other hand, the levels of MGP mRNA were decreased in accordance with the increase of calcium deposition and at 10 mM β-GP the expression was maximally depressed to 47.6% of uncalcified control (Fig. 2b). These results suggest that the expression of MGP gene is inversely correlated with the degree of BVSMC calcification.

Finally, we examined the effects of bisphosphonates such as etidronate (EHDP) and alendronate on the expression of MGP mRNA. Both of EHDP and alendronate dose-dependently inhibited BVSMC calcification (Figs. 3a and 4b, respectively). In the presence of EHDP or alendronate, MGP mRNA expression was restored to the level of uncalcified control (Figs. 3b and 4b, respectively). These data further confirm that the expression of MGP gene is modulated in the development of BVSMC calcification.

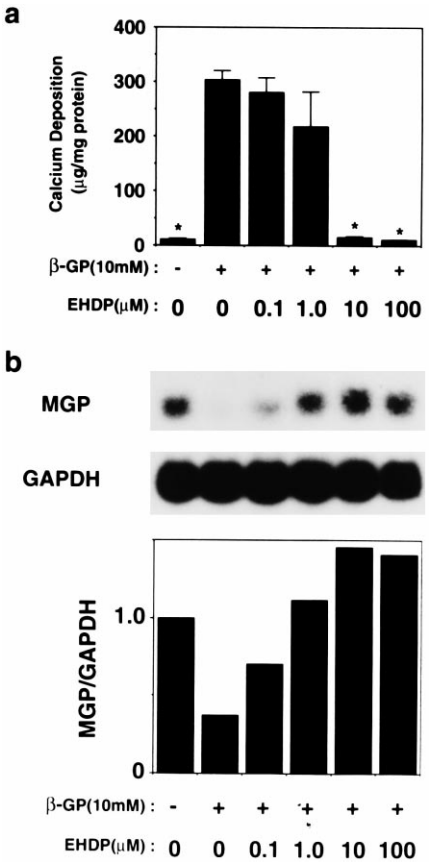

Fig. 3. Dose-dependent effects of etidronate on BVSMC calcification (a) and expression of MGP mRNA (b). The cells were cultured for 4 days as described in Section 2 in the presence of the indicated concentrations of etidronate (EHDP). a: The calcium content was measured by *o*-cresolphthalein complexone method, normalized by cellular protein content, and is presented as mean ± S.E.M. The differences compared with calcified control were statistically significant (*$P < 0.05$, Fisher's PLSD). b: Twenty micrograms of total RNA from BVSMC were electrophoresed, blotted, and probed with cDNA of bovine MGP. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Upper panel, autoradiograph of Northern analysis of MGP. Lower panel, densitometric analysis of the autoradiograms was performed and the results are presented as the ratio of MGP to GAPDH.

4. Discussion

We reported here for the first time the inverse relationship between vascular calcification and expression of MGP gene by using an in vitro model of vascular calcification. Our results indicate that vascular calcification may modulate the expression of MGP gene. MGP is a vitamin K-dependent protein and belongs to a family of extracellular mineral-binding proteins called Gla proteins [1–3]. Gla residues have high affinity for calcium and phosphate ions, and hydroxyapatite crystals [2]. Unlike bone Gla protein, which may be involved in mineralization of skeletal tissues, the function of MGP remains unknown. MGP was first isolated from bovine bone, but is now known to be synthesized in various tissues including vascular smooth muscle cells [4,12]. Furthermore, Shanahan et al. reported that MGP and osteopontin were highly expressed in human atherosclerotic plaques, suggesting that bone matrix proteins, such as MGP, have an important role in the pathogenesis of atherosclerotic plaques [8]. More recently, it is reported that MGP-deficient mice developed arterial calcification and died within 2 months by blood vessel rupture [6]. These results strongly suggest that MGP may function as an inhibitor of arterial walls. However, whether MGP itself prevents vascular calcification in this model remains to be clarified.

Vascular calcification is often detected in advanced atherosclerotic lesions and was generally regarded as an unregulated and end-stage process of atherosclerosis. However, it has been recently becoming recognized that it is an active and regulated process similar to osteogenesis, since bone matrix proteins, such as osteopontin and MGP are expressed in atherosclerotic lesions [8]. Moreover, vascular calcification is also often associated with cartilaginous metaplasia in which MGP seems to be abundantly expressed [13]. Therefore, it is likely that MGP may modulate the osteogenic and/or chondrocytic differentiation of VSMC in the development of atherosclerotic lesions. Additionally, it is noteworthy that MGP-deficient mice have

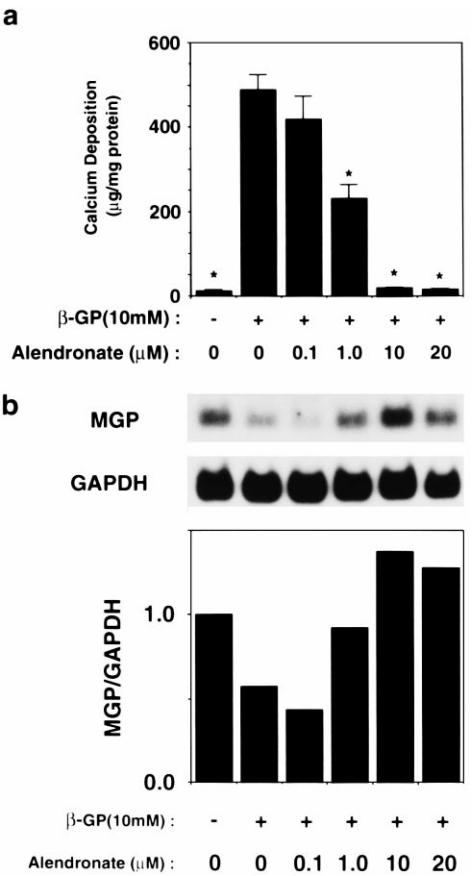

Fig. 4. Dose-dependent effects of alendronate on BVSMC calcification (a) and expression of MGP mRNA (b). The cells were cultured for 4 days as described in Section 2 in the presence of the indicated concentrations of alendronate. a: The calcium content was measured by *o*-cresolphthalein complexone method, normalized by cellular protein content, and is presented as mean ± S.E.M. The differences compared with calcified control were statistically significant (*$P < 0.05$, Fisher's PLSD). b: Twenty micrograms of total RNA from BVSMC were electrophoresed, blotted, and probed with cDNA of bovine MGP. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Upper panel, autoradiograph of Northern analysis of MGP. Lower panel, densitometric analysis of the autoradiograms was performed and the results are presented as the ratio of MGP to GAPDH.

arterial calcification but no evidence of atherosclerotic plaques [6], suggesting that vascular calcification may be controlled by the genes including MGP, which are different from the genes regulating the formation of atherosclerotic lesions.

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