Assay for Human Matrix Gla Protein in Serum
Potential Applications in the Cardiovascular Field


Abstract—Matrix Gla protein (MGP) is synthesized in a vitamin K–dependent way in smooth muscle cells of the healthy vessel wall, and its mRNA transcription is substantially upregulated in atherosclerotic lesions. Here we report the preparation of a monoclonal antibody against human MGP and its use in an enzyme-linked immunosorbent assay. The intra-assay and interassay coefficients of variation in serum samples were 5.4% and 12.6%, respectively, and the lower detection limit was 8.5% of the normal serum value. Individual within-day variations were <11% and did not show a distinct circadian pattern. Day-to-day variations in fasting morning samples were <8%. In a first explorative survey, serum MGP concentrations were found to be significantly increased in patients with severe atherosclerosis, whereas these values were normal in those with low bone mass and osteoporosis. This finding is consistent with the high MGP mRNA expression observed in atherosclerotic vessels and plaques. More elaborate studies are required to assess the potential clinical utility of this newly developed assay. (Arterioscler Thromb Vasc Biol. 2000;20:1257-1261.)

Key Words: vitamin K \(\gamma\)-carboxyglutamate \(\gamma\)-carboxylation atherosclerosis ectopic calcification diagnostics

Vitamin K is a cofactor in the posttranslational conversion of glutamate residues into \(\gamma\)-carboxyglutamate (Gla). At this time, 10 mammalian Gla-containing proteins have been described in detail, and the number of Gla residues per molecule varies from 3 (osteocalcin [OC]) to 13 (protein Z). In all cases in which their function is known, the activity of the various Gla proteins is strictly dependent on the presence of the Gla residues.\(^1\) One of the Gla proteins is matrix Gla protein (MGP); it is synthesized by chondrocytes and vascular smooth muscle cells.\(^2,3\) Small amounts of MGP mRNA have also been detected in various other tissues,\(^4\) but this may reflect, at least in part, synthesis in small vessels and capillaries. Although its mode of action on a molecular level has remained obscure until now, recent data in rodents strongly suggest that MGP plays a key role in the inhibition of tissue calcification. MGP-deficient mice were generated by Luo et al,\(^5\) who observed excessive cartilage and growth plate mineralization, resulting in impaired growth of the long bones. An even more prominent phenomenon, however, was that all animals showed massive calcification of the main arteries and died within 8 weeks after birth due to rupture of the thoracic or abdominal aorta. The importance of Gla residues for MGP to exert its mineralization-inhibitory function was demonstrated in rats in which extrahepatic protein carboxylation had been blocked by treatment with warfarin.\(^5\) After 3 to 4 weeks of treatment, these animals developed arterial calcifications starting around the elastic lamellae of the media in a similar way as was reported for MGP-deficient mice. Whether this effect was due to poor cellular excretion of undercarboxylated MGP or its lack of functionality has remained unclear in that experiment. It is generally assumed, however, that in MGP, as in other Gla proteins, the Gla residues are important for its function. Taken together, the available data in rodents demonstrate that MGP is a potent inhibitor of tissue calcification and that its posttranslational carboxylation is essential for exerting this activity in vivo. A recent publication by Munroe et al\(^6\) has suggested that vascular calcification in humans may be more complex than in rodents. These authors reported data for 3 unrelated patients with Keutel syndrome (KS), which is an autosomal recessive disorder characterized by abnormal cartilage calcification. These authors showed that the 3 KS patients had (different) mutations in their MGP genes that predicted a frameshift or a premature frameshift in the mature protein. KS patients may therefore be regarded as human models for MGP deficiency, but remarkably, arterial calcification is not a common feature in human KS. It should be noted, however, that neither a histological nor a pathological examination of the arteries was performed in the patients described, so the effect of MGP deficiency on human vascular biology remained to be investigated.

Cardiovascular disease is 1 of the major life-threatening diseases in Western society, but biomarkers to monitor the severity or progression of the disease are presently unavail-
able. Also, the number of biochemically detectable risk factors (eg, serum cholesterol, triglycerides, apoE genotype) is surprisingly low. Based on the limited data available, serum MGP is a good candidate to become a biomarker associated with arterial calcification. In this article, we report the production of a monoclonal antibody (mAb 8–15) against human MGP and the development of a microtiter plate-based assay with which circulating MGP levels were demonstrated and quantified in human serum. The assay may be used to explore the potential value of circulating MGP as a marker in the field of cardiovascular disease. Moreover, it may help us to understand the role of MGP in human vascular biology.

Methods

Materials

Synthetic peptides homologous to the sequences 3 to 15 and 63 to 75 of human MGP and to the sequences 1 to 16 and 29 to 43 of human OC were synthesized and purified by Pepscan Systems (Lelystad, The Netherlands) and for purpose of this discussion will be designated as MGP3–15, MGP61–75, OC3–16, and OC63–75, respectively. All chemicals used were of analytical or high-performance liquid chromatography grade.

Preparation of Recombinant (r) MGP

mRNAs coding for MGP and OC were isolated from cultured osteoblasts and used for preparing the corresponding cDNAs. Both cDNAs were inserted into the pQE-40 vector (Qiagen) and expressed in Escherichia coli M15 as chimeric proteins with murine dihydrofolate reductase (DHFR) equipped with an N-terminal 6-His tag for rapid purification (H.M.H.S., unpublished data, 1999). After expression, bacteria were lysed in buffer A (8 mol/L urea, 0.3 mol/L NaCl, and 0.01 mol/L Tris-HCl, pH 8.0). After centrifugation for 30 minutes at 10 000 g, the supernatant was passed over N+ nitriilotriacetic acid agarose column (Qiagen) in buffer A, and the 6-His–tagged protein was eluted with buffer B (8 mol/L urea, 0.5 mol/L imidazole, pH 8.0). Unfortunately, the preparation thus obtained was insoluble under physiological conditions, which hampered its use as a reference material in the MGP assay.

Preparation of Antibodies

BALB/c mice were immunized intraperitoneally with the peptide MGP3–15, which was coupled to keyhole limpet hemocyanin (Pierce Chemical Co). Twenty micrograms of antigen in Freund’s complete adjuvant was used for the first immunization, followed by 3 boosts (20 μg each) in Freund’s incomplete adjuvant given at 2-week intervals. Postimmune sera were screened for their affinity toward purified rMGP, which was used as a chimeric construct with murine DHFR (see below). At 1 week after the last boost, splenocytes of the best responder mouse were fused with an American Type Culture Collection (Manassas, Va) mouse myeloma cell line (Sp 2/0-Ag, CRL 8006) according to standard procedures, and growing hybridomas were screened by an ELISA in which recombinant proteins were coated to the microtiter plate. Positive clones were selected on the basis of specific DHFR-MGP recognition, whereas rDHFR-OC served as a negative control. A clone with a strong and specific reaction with rMGP was selected for the large-scale preparation of monoclonal antibodies (mAb 8–15). In a final step, the IgG was isolated from the culture medium by protein G affinity chromatography.

MGP Assay

Urea-solubilized rMGP (1 g/L) was diluted 50-fold with coating buffer (0.1 mol/L sodium carbonate, pH 9.6) and used for the coating of microtiter plates (50 μL/well). After incubation for 1 hour at 37°C, remaining protein-binding sites were blocked with 100 μL/well of blocking buffer (Hoffmann–La Roche; catalog No. 1 112 589) incubated for another 1 hour at 37°C. After thorough washes (with washing buffer, consisting of 0.3%/wt/vol Tween-20 in PBS [0.15 mol/L NaCl, 10 mmol/L sodium phosphate, pH 7.4]), the plates were ready for use. The serum samples were diluted as indicated with PBS, and 125 μL of sample was supplemented with 25 μL of mAb 8–15 (6 mg/L in PBS containing 2%/wt/vol nonfat dry milk protein [Nutricia]) and incubated for 5 minutes at room temperature. Subsequently, 50 μL of sample was transferred to the microtiter plate and incubated for 1 hour at 37°C. After 3 washing cycles with the PBS–Tween-20 washing buffer (see above), the mAb 8–15 bound to the plate was quantified by using a second antibody (rabbit anti-mouse total IgG conjugated with horseradish peroxidase [Dako; 1 mg/L in PBS–Tween-20]) and stained with 3,3′,5,5′-tetramethylbenzidine (Hoffmann–La Roche). After 10 minutes, the staining process was stopped by adding 200 μL of 1 mol/L H2SO4, and the plate was read at 450 nm. The MGP content of pooled reference sera from 30 healthy individuals was arbitrarily defined to be 100 U/L.

Subjects

Unless stated otherwise, fasting blood samples were taken. Serum was left at room temperature for 2 hours before centrifugation (15 minutes, 2000g) and storage at –80°C until use. For assessment of the normal range and reference groups, apparently healthy subjects were recruited from the general Maastricht population. The day-to-day and within-day variations were determined in a group of 12 healthy men (20 to 35 years old), from whom blood was taken by venipuncture at 9 time points on 1 day and on 4 different days at 9 AM at 1-week intervals. Samples were also obtained from 200 healthy subjects (55 to 65 years old) in whom the intima/media thickness of the carotid artery had been measured by ultrasound as described by Hoeks and Reneman. Patient samples were obtained from the University Hospital Maastricht. The study was approved by the local Medical Ethics Committee, and informed consent was obtained from all participants, according to institutional guidelines.

Statistical Analysis

The Student's t test (for groups of n≥30) and the Mann-Whitney U test (for groups of n<30) were performed to assess whether observed differences between groups were statistically significant (P<0.05).

Results

Calibration Curve and Test Characteristics

Calibration curves were made on 12 different days by using 6 different dilutions of pooled reference serum. Each dilution was measured in duplicate, and the mean optical densities at 450 nm (±SD) were expressed as a function of serum concentration (Figure 1A). At increasing dilutions of the serum sample, more anti-MGP was bound to the plate, with the buffer value as a theoretical maximum. The lower
Validation of the assay was performed in a number of control experiments, which are summarized in Figure 1B. To eliminate the possibility of a false-positive signal because of cross-reaction of the second antibody with microtiter-bound proteins, the assay was performed in the absence of mAb3–15. No response was obtained under these conditions. To eliminate the possibility that human serum contains autoantibodies against MGP that might interfere with the assay, serum was transferred in 7 subsequent steps across microtiter plate wells coated with rMGP before it was used in the MGP assay. Dilution curves of sera with and without this pretreatment were identical, thus denying the occurrence of preexisting anti-MGP. Next, we investigated whether human test samples might contain IgG that would interfere with the assay by binding directly to mouse IgG. To this aim, serum was analyzed in various dilutions before and after adsorption onto protein G–Sepharose. Both curves were identical, thereby showing that the assay was not disturbed by preexisting anti-murine IgG.

Sample Preparation
To further evaluate the robustness of the assay, we checked the influence of variations in the sample preparation procedure at the following steps: centrifugation speed (1500 and 10 000g) during serum preparation, centrifugation (10 000g) after addition of mAb3–15, freeze-thawing of the serum sample (up to 8 cycles of freeze-thawing), and incubation time (between 3 and 60 minutes at room temperature) of the serum sample with mAb3–15. In none of these cases did the sample treatment measurably affect the observed MGP concentration.

Assay Specificity
The mAb3–15 used in the assay was tested for its ability to differentiate between 2 recombinant bone Gla proteins: OC and MGP (both as chimeric constructs linked with 6-His–DHFR). Microtiter plates were coated with either purified rMGP (1 μg/well) or equimolar amounts of purified OC. The coupling efficiency of both proteins was checked with anti–6-His antibodies. As shown in Figure 2, both plates contained similar amounts of recombinant protein (MGP in A and OC in B), and mAb3–15 reacted well with MGP but not with OC. The species specificity of mAb3–15 was tested further by comparing its reaction with human, rat, and murine sera. Cross-reaction with rodent sera was below the detection limit (<8.5 U/L) at all dilutions tested. Epitope specificity was tested by comparing the extent to which various synthetic peptides were capable of extinguishing the response with 10-fold–diluted human serum. Under standard conditions (ie, when 6.7 nmol/L mAb3–15 was used), almost complete quenching of the signal was obtained by mixing the serum with 50 nmol/L MGP3–15, with a half-maximal effect at 5 nmol/L. No effect was observed with the peptides MGP63–75, OC1–16, and OC96–105 up to concentrations of 65 μmol/L (see also Figure 3).

Normal Range, Within-Day Variations, and Day-to-Day Variations
The “normal range” for MGP was established in 80 apparently healthy men between 20 and 84 years of age. It was found that the mean value for serum MGP in this group was 96±17 U/L. Hence, the normal range (defined as the mean±2SD) was calculated to be between 62 and 130 U/L. No apparent age dependence was observed for MGP in this group. Similar data were observed for elderly women (>60 years of age), but a larger range was found in women between 20 and 55 years old. This may be related to hormonal changes and forms the basis for our decision that women <60 years old were not included in the experiments presented in this article. The time-related variability of serum MGP was established in a group of 12 healthy subjects from whom blood was taken by venipuncture at 9 time points on 1 day and on 4 different days at 9 AM at 1 week intervals. The within-day variation was calculated for each subject separately by expressing the SD as a percentage of the mean of the
9 time points and amounted to 11%. No distinct circadian pattern was observed (see Figure 4). The day-to-day variation was calculated in a similar way from the 4 samples obtained at weekly intervals, and was found to be 8%.

**MGP in Patients**

The potential clinical utility of the newly developed MGP assay was tested in a pilot study among a limited number of patients. Because bone and the arterial vessel wall are the major sites of MGP production, we focused on subjects with either bone disease (osteoporosis) or vascular disease (atherosclerosis), and these data are summarized in the Table. No correlations were found between serum MGP levels and either low or high bone mass, osteoporosis, or vascular intimal thickening. On the other hand, circulating MGP was significantly elevated in subjects with advanced atherosclerosis. Also, those with type I diabetes mellitus, a risk factor for atherosclerosis, had increased circulating MGP levels.

**Discussion**

In this article, we report the development of an assay for human MGP and, with this assay (a so-called antibody-capture ELISA), the presence of MGP-related antigen in the circulation was established. Recorded immunoreactive MGP turned out to be independent of sample preparation and showed small within-day and day-to-day fluctuations. At this time, the assay still has a number of weaknesses inherent to a single-antibody assay. Substantial improvements may be expected from the availability of a second antibody, which will allow us to set up a sandwich ELISA. The question remains concerning the origin of serum MGP. The protein is known to be synthesized by chondrocytes in the cartilage and by smooth muscle cells in the arterial vessel wall. In a first survey among a limited number of patients, we have demonstrated that in severely atherosclerotic patients, circulating MGP was significantly increased. In more elaborate clinical studies, it should be investigated whether the severity of the disease (eg, the extent of aortic calcification) is correlated with the concentration of serum MGP, but at this time no such data are available. In contrast to the data obtained in atherosclerotic subjects, MGP was found to be normal in all cases of bone disease tested thus far. The apparent association between serum MGP and vascular disease suggests that the circulating protein originates from the vessel wall rather than from bone.

In bone, MGP accumulates in relatively large quantities, which is why bone is the only tissue from which native MGP has been isolated thus far. However, under physiological conditions, MGP originating from human and bovine bone is 1 of the most insoluble proteins known. Comparison between its primary structure and the amino acid sequence derived from cDNA coding for MGP shows that in bone-derived MGP, the last 7 C-terminal amino acids are missing, and it may be imagined that proteolytic cleavage of its C-terminus forms a mechanism for insolubilizing MGP, by which it is retained in bone tissue. On the other hand, the possibility cannot be excluded that in bone, MGP is complexed with the organic or inorganic matrix or that it is folded in such a way to prevents its escape into the circulation. Because of its poor solubility, it is difficult to envisage how significant amounts of MGP could be filtering from bone into the circulation.

From in situ hybridization, we also know that MGP mRNA transcription takes place in the arterial vessel wall, but with the aid of immunohistochemical techniques, only low levels of MGP protein have been found in the healthy vessel wall. Thus, unlike bone and cartilage, healthy vessels do not retain considerable stores of MGP. The reported strong upregulation of MGP mRNA synthesis and the large amounts of immunoreactive MGP found at sites of atherosclerotic lesions suggest a feedback mechanism for local synthesis of MGP-related antigen, the Gla content and calcification inhibitory activity of which remain unknown. This concept is consistent with the hypothesis that at least part of the vascular MGP reaches the circulation and may account for the positive signal obtained in healthy subjects and for the elevated serum values observed in atherosclerotic patients. Our hypothesis does not explain the mechanism by which circulating MGP remains in solution. One possibility is that after cellular secretion, vascular MGP is processed differently from that in bone. From its primary structure, it can be deduced that among the last 7 amino acids predicted by the cDNA sequence coding for the 84 residues of human MGP, 5 are positively charged. These 7 C-terminal amino acids are missing in MGP isolated from bone but may be present in serum MGP. Hence, the isolation and C-terminal sequence determination of serum MGP may provide evidence for its origin. An alternative explanation for the apparent solubility

**Serum MGP Concentrations in Patients**

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Serum MGP (% of Age- and Sex-Matched Controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High femur BMD (&gt;mean + ISD)</td>
<td>40</td>
<td>98.5±5.7</td>
</tr>
<tr>
<td>Low femur BMD (&lt;mean – ISD)</td>
<td>38</td>
<td>102.0±5.2</td>
</tr>
<tr>
<td>Senile osteoporosis</td>
<td>28</td>
<td>101.8±6.8</td>
</tr>
<tr>
<td>Increased intima/media thickness</td>
<td>43</td>
<td>97.3±6.1</td>
</tr>
<tr>
<td>Type 1 diabetes mellitus</td>
<td>23</td>
<td>134.5±8.6*</td>
</tr>
<tr>
<td>Severe atherosclerosis</td>
<td>26</td>
<td>145.8±6.6*</td>
</tr>
</tbody>
</table>

Data are given as mean±SE. Increased intima/media thickness was the highest quartile from a group of 200 apparently healthy elderly subjects. Subjects with high and low bone mass density (BMD) of the femur neck were obtained from a reference population (N=250) recruited from the general Maastricht population. Patients with osteoporosis, diabetes mellitus, and atherosclerosis were obtained from various departments of the University Hospital Maastricht.

Values indicated by an asterisk were significantly different from the controls (P<0.05).
of serum MGP is that it may be bound to a soluble carrier protein or that it is associated with the lipoprotein fraction.

Assuming that the Gla residues in MGP are essential for either its cellular secretion or its calcification inhibitory activity, poor vitamin K status could be an independent risk factor for tissue calcification. The latter hypothesis is consistent with data from Jie et al., who demonstrated in a population-based study (EPOZ) an inverse correlation between dietary vitamin K intake and the occurrence of calcified aortic lesions in elderly subjects. The fact that major fractions of both OC and MGP isolated from human bone as well as circulating OC seem to occur in an undercarboxylated form suggests that the human vitamin K requirement should not be inferred from the hepatic synthesis of fully carboxylated coagulation factors but from the ability of nonhepatic tissues to maintain full carboxylation of locally produced proteins such as OC and MGP. All presently available data suggest that the extrahepatic Gla proteins are more susceptible to a reduced dietary intake of vitamin K than are the classic coagulation factors and that present recommended daily allowance values should be redefined to ensure complete carboxylation of extrahepatic Gla proteins. Unfortunately, the assay described in this article does not discriminate between carboxylated and undercarboxylated MGP, which hampers full evaluation of its diagnostic value. More elaborate clinical studies are required to evaluate whether MGP total antigen may become a marker for diagnosis or patient follow-up during treatment of atherosclerosis.

Acknowledgments

This study was supported in part by grant No. 28-2817 from the Prevention Fund and by Hoffmann–La Roche Diagnostics (Kaiseraugst, Switzerland). The authors wish to thank M. Henfling for her skilled help in monoclonal antibody production.

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Arterioscler Thromb Vasc Biol. 2000;20:1257-1261
doi: 10.1161/01.ATV.20.5.1257

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