The Putative RGD-dependent Cell Adhesion Activity of Matrix Gla Protein Is Due to Higher Molecular Weight Contaminants

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This study was carried out to further characterize the RGD-dependent cell adhesion activity which was previously observed in the vitamin K-dependent matrix Gla protein (MGP) (Loeser, R. F., and Wallin, R. (1992) J. Biol. Chem. 267, 9459-9462). We have found that this cell adhesion activity can be completely removed from the 10-kDa MGP by gel filtration over Sephacryl S-200-HR. The higher molecular weight contaminants removed by the gel filtration step display potent cell adhesion activity. The additional evidence previously adduced in support of the putative cell adhesion activity of MGP is that heat decarboxylation of the vitamin K-dependent γ-carboxyglutamate residues in MGP abolished the adhesion activity. The heat decarboxylation conditions used, however, appear to cause other chemical changes in proteins in addition to decarboxylation, as evidenced by the fact that the cell adhesion activity of fibronectin, which is not a vitamin K-dependent protein, is also destroyed by this procedure.

The present evidence that the putative cell adhesion activity of MGP is caused by contaminating higher molecular weight cell adhesion proteins accounts for two apparent anomalies in the previously reported cell adhesion activity of MGP, the failure of antibodies raised against a synthetic peptide corresponding to the C-terminal 19 residues of bovine MGP to inhibit the cell adhesion activity of the intact, 79-residue bovine protein, and the potent inhibition of the cell adhesion activity of MGP by a synthetic peptide containing an RGD sequence, even though MGP does not contain this sequence.

Matrix Gla protein (MGP)1 is a 10-kDa vitamin K-dependent protein which is synthesized by an exceptionally broad array of tissues and cells (1-7). MGP mRNA has been found by Northern blot in all vertebrate tissues tested to date, with the highest levels in heart, lung, kidney, and cartilage (3, 4). MGP is also secreted by many cells in culture, including osteoblasts, fibroblasts, chondrocytes, myocytes, vascular smooth muscle cells, breast cells, pneumocytes, and kidney cells (3-7). In spite of the broad tissue distribution of MGP synthesis, only three tissues have been found to accumulate significant levels of MGP in an extracellular matrix, bone, cartilage, and calcified cartilage (1, 4, 5).

Although the function of MGP is presently unknown, there is evidence to suggest that the protein could regulate an aspect of cell growth and differentiation. MGP gene transcription is strongly induced by retinoic acid in all normal human cell types tested (5), and the human MGP gene promoter contains a perfect direct repeat which is nearly identical to the retinoic acid response element in the human retinoic acid receptor β gene promoter (9). These observations suggest that MGP could mediate some of the known actions of retinoic acid on cell growth and differentiation (5). Following the original isolation and sequencing of MGP from bone (1, 2), MGP has been independently discovered by differential cDNA screening as a gene which is overexpressed by breast cancer cells (6), by prostate epithelial cells undergoing apoptosis (10), and by vascular smooth muscle cells undergoing dedifferentiation in cell culture (11). Although each of these three independent discoveries of MGP is based on increased MGP production in cells undergoing fundamentally different transitions, the fact that MGP is one of the few genes overexpressed in each instance does suggest that MGP expression is driven by transitions in cell growth and differentiation and that the protein may in fact play a role in these processes.

The only in vitro MGP activity reported to date is a potent cell adhesion activity for fibroblasts, osteoblasts, chondrocytes, and kidney mesangial cells (12, 13). The putative cell adhesion activity of MGP is unusual in two respects. The cell adhesion activity of bovine MGP cannot be inhibited by high concentrations of polyclonal antibodies raised against a synthetic 19 residue peptide which corresponds to the C terminus of the 79-residue bovine MGP (2, 12). In addition, the MGP cell adhesion activity is strongly inhibited by a peptide which contains the RGD sequence (12), even though the bovine MGP tested does not itself contain this sequence (2). Human, rat, mouse, and shark MGP also lack an RGD sequence (8, 9, 14, 15).

The present studies were carried out to further characterize the cell adhesion activity of bovine MGP. We report here evidence that the putative cell adhesion activity of MGP is in fact due to higher molecular weight contaminants and that MGP itself is devoid of cell adhesion activity.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, cytochrome c, and aprotinin were purchased from Sigma. Bovine fibronectin was purchased from CalBiochem. The cell adhesion assays were performed using Nunc Maxisorp plates. The level of MGP antigen in effluent fractions was determined by radioimmunoassay (19) on aliquots of 5 ul or less. Minimal MGP levels...
were found in the higher molecular weight contaminants (Fig. 1); these levels were: pool A, 0.5 μg MGP/ml (0.3% of protein); pool B, 1.6 μg MGP/ml (1.4%); pool C, 5.5 μg MGP/ml (2.1%); and pool D, 8.4 μg MGP/ml (4.8%). The MGP peak fractions (Fig. 1) were either stored frozen at -70 °C or dialyzed against 50 mM HCl and freeze-dried. MGP and fibronectin were subjected to conditions known to decarboxylate γ-carboxyglutamate residues to glutamate (17, 18) by freeze-drying the respective proteins from 50 mM HCl and subsequently heating the dry proteins for 8 h at 110 °C in evacuated sealed tubes.

Cell Culture—Human foreskin dermal fibroblasts and human articular cartilage chondrocytes were cultured in medium containing 10% fetal calf serum as described (5).

Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed under reducing conditions as described (4) using precast 4–20% gradient polyacrylamide gels (Novel Experimental Technology, San Diego, CA), run at a constant 150 volts. Protein bands were visualized by staining the gel for 90 min with 0.2% Coomassie Blue R-250, 10% trichloroacetic acid, and 10% 5-sulfosalicylic acid and destaining overnight in 6% methanol, 7.5% glacial acetic acid in water. After electrophoresis, proteins were electroblotted onto nitrocellulose and Western blot analysis was performed using anti-bovine fibronectin and anti-bovine vitronectin antisera (Life Technologies, Inc.) at a 1:1000 dilution.

Cell Adhesion Assay—Cell adhesion assays were carried out in 96-well microtiter plates which had been previously coated with the desired concentration of each test protein. Because MGP has a low solubility in the absence of denaturants (1, 2), stock solutions of different MGP preparations were first prepared at a concentration of 5 and 25 mg of protein/ml in 4 M guanidine HCl with 50 mM Tris, pH 7.6, and then diluted into phosphate-buffered saline (PBS) to give the desired final MGP concentration in the coating solution; this procedure was used successfully in the previous studies (12). Radioimmunoassay (19) of coating solutions before and after incubation in the microtiter wells confirmed that the desired MGP concentrations were in fact initially present in the coating solutions and that MGP did adsorb to the microtiter wells. All other proteins tested in cell adhesion assays were dissolved in PBS and diluted in PBS to the desired protein concentration. Each microtiter well was coated with 200 μl of the desired concentration of protein in PBS by incubation in a humidified incubator at 37 °C overnight. The coating solution was then removed, and the microtiter wells were washed three times with 200 μl of PBS and then blocked with 100 μl of 3% bovine serum albumin in PBS for 1 h at 37 °C. After blocking, the wells were washed once with 200 μl of serum-free medium and then received 100 μl of serum-free culture medium followed by the same volume of serum-free medium containing either human dermal fibroblasts or human articular cartilage chondrocytes at a concentration of 10^6 cells/ml. The final cell density was therefore 10^5 cells/well. Plates were incubated for 30–90 min at 37 °C in the humidified incubator and then examined with an inverted microscope for evidence of cell attachment and spreading. Medium was removed by inverting the plate and pressing the surface to absorbent paper towels. Each well then received 200 μl of PBS followed by inversion and medium removal for a total of three washes. Wells were inspected in an inverted microscope to assess

**Fig. 1.** Sephacryl S-200-HR purification of bovine MGP. Bovine MGP was extracted from bone and purified by ion exchange chromatography as described (16). This purified MGP preparation was then dissolved in 6 M guanidine HCl with 100 mM Tris, pH 9.0, and applied to a 2 x 150-cm Sephacryl S-200-HR column equilibrated in the same buffer at 22 °C. The absorbance at 230 nm was determined for each 4-ml fraction (○), and the level of MGP antigen was measured by radioimmunoassay on aliquots of 5 μl or less (■) (see "Experimental Procedures"). Inset, SDS-polyacrylamide gel electrophoresis of effluent fractions. The indicated fractions were pooled, dialyzed against 50 mM HCl, dried, electrophoresed on a 4–20% gel, and stained with Coomassie Blue (see "Experimental Procedures"). Lane 1, Bio-Rad high molecular weight standards; lane 2, 20 μg of pool A (fractions 48–54); lane 3, 20 μg of pool B (fractions 55–57); lane 4, 20 μg of pool C (fractions 58–61); lane 5, 20 μg of pool D (fractions 62–64); lane 6, 20 μg of MGP (fractions 68–73); lane 7, Bio-Rad low molecular weight standards.
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cell attachment and spreading and then subjected to the colorimetric
hexosaminidase assay using described procedures (20).

Cells were prepared for adhesion assays from confluent cultures of
normal human dermal fibroblasts at passages 8-12 and from confluent
cultures of normal human articular cartilage chondrocytes at passage 1
by trypsin disaggregation of the cells, termination of trypsin action by
the addition of 5 ml of medium containing 1% serum, disaggregation of
cells by pipetting, sedimentation of cells by centrifugation, two succes-
sive washes of the cells with 50 ml of medium containing 0.05% serum,
and suspension of cells in 20 ml of serum-free medium. Cells were
counted and then sedimented and resuspended at the desired final
concentration of 10^4 cells/ml in serum-free medium. Cells were allowed
to recover for 30 min at 37 °C before addition to the microtiter plate for
the cell adhesion assay.

Antibody Purification—Rabbit antibodies against bovine MGP were
purified by affinity chromatography (4) using Sepharose 4B to which
MGP had been attached by the cyanogen bromide procedure. The MGP
employed in this purification was purified by size fractionation and was
devoid of cell adhesion activity. A portion of these affinity-purified an-
tibodies was subsequently attached to Sepharose 4B by the cyanogen
bromide procedure.

RESULTS

Effect of Size Fractionation on the Cell Adhesion Activity of
Matrix Gla Protein Preparations—The MGP preparation pre-
viously found to have potent cell adhesion activity for normal
human dermal fibroblasts and other cells (12) was purified
from bone by extraction and ion exchange procedures we de-
veloped (16), which do not include a size fractionation step.
Since most known cell adhesion proteins are larger than the
10-kDa MGP, we first fractionated this MGP preparation by
size on a Sephadex S-200-HR column to remove possible higher
molecular weight contaminants before testing its cell adhesion
activity. As can be seen in Fig. 1, higher molecular weight
protein contaminants are in fact removed from the MGP prepa-
ration by this procedure. Based on absorbance at 230 nm, these
higher molecular weight contaminants account for approxi-
mately 20% of the protein recovered from the column, whereas
MGP itself accounts for 80%. Radioimmunoassay for MGP (19)
revealed that the MGP immunoreactivity was recovered in the
single MGP peak and that essentially no MGP immunoreactiv-
ity could be detected in the higher molecular weight protein
components (Fig. 1). The MGP purified by this size fraction-
ation step was free of high molecular weight contaminants as
judged by Coomassie staining of 4–20% gradient SDS-polyac-
rylamide gel electrophoresis (inset, Fig. 1).

Prior to size fractionation, we found that MGP preparations
have cell adhesion activity for human fibroblasts which is, on a
weight basis, even stronger than fibronectin (Fig. 2), in agree-
ment with the cell adhesion activity reported previously for this
MGP preparation by Loeser and Wallin (12). After size fractiona-
tion, however, MGP is totally devoid of detectable cell adhe-
sion activity (Fig. 2). The complete absence of cell adhesion
activity in MGP purified by size fractionation was observed in
over 20 separate cell adhesion experiments conducted on sev-
eral different purification batches of MGP over a 5-month pe-
riod and using MGP coating concentrations of up to 100 μg/ml.
In each of these experiments, visual inspection of cell adhesion
using an inverted phase-contrast microscope correlated with
the hexosaminidase assay results, and MGP purified by size
fractionation was never able to promote cell attachment or
spreading. The loss in the cell adhesion activity of MGP upon
size fractionation cannot be explained by irreversible denatur-
ation of the protein, which could in theory be caused by the 6 M
guanidine HCl buffer used in the size fractionation step, since
the dose dependence and magnitude of the cell adhesion activ-
ity found in MGP prior to size fractionation is not affected by
incubation for 3 days at 22 °C in 6 M guanidine HCl (data not
shown). It should also be noted that the MGP preparation,
which does have potent cell adhesion activity, has in fact al-
ready been exposed to denaturation conditions during its puri-
fication, since the buffer used in the ion exchange chromatog-
raphy step contains 8 M urea (12, 16). The highest molecular
weight contaminants removed from MGP by size fractionation
(fractions 48–54 in Fig. 1) are potent in the cell adhe-
sion assay (Fig. 3). SDS-polyacrylamide gel electrophoresis
revealed the existence of several high molecular weight proteins
in this active fraction (Fig. 1), the most prominent of which had
apparent molecular masses of 127 and 147 kDa. Analysis of these
highest molecular weight MGP contaminants by immu-
noblot revealed the presence of two known cell adhesion mol-
ecules, bovine fibronectin and bovine vitronectin, and it is pos-
sible that some or all of the cell adhesion activity found in the
MGP contaminant fraction could be explained by the presence
of these proteins.

Similar results were obtained when the cell adhesion activity
of MGP preparations was tested using human articular carti-
gle chondrocytes rather than human fibroblasts. When tested
at a coating concentration of 10 μg of protein/ml, the MGP
preparation prior to size fractionation had potent cell adhesion
activity for chondrocytes, whereas MGP obtained after size
fractionation was devoid of detectable cell adhesion activity
(data not shown). In addition, the highest molecular weight
contaminants removed from MGP by size fractionation (frac-
tions 45–54 in Fig. 1) had strong cell adhesion activity for
chondrocytes.

Effect of Conditions Used for the Decarboxylation of Gla to
Glu on the Cell Adhesion Activity of Fibronectin—In the previous
report (12) that MGP has cell adhesion activity, one of the
major lines of evidence presented was the finding that the cell
adhesion activity of the MGP preparation used is inactivated by
the heat decarboxylation of the vitamin K-dependent amino
acid residues in MGP, γ-carboxyglutamic acid, to glutamic acid.
The procedure used, heating protein dried from 50 mm HCl for
8 h at 110 °C, was developed in our laboratory and does indeed
decarboxylate γ-carboxyglutamic acid residues to glutamic acid
(17, 18). There is no published report, however, that proteins
which do not contain γ-carboxyglutamic acid will in fact retain
activity when exposed to these acid decarboxylation conditions
(17). When fibronectin was treated with conditions identical to
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FIG. 3. Effect of decarboxylation on cell adhesion to fibronectin. Microtiter plates were coated overnight with 1 μg/ml concentrations of: bovine serum albumin, native fibronectin (FN), heat-"decarboxylated" fibronectin (DeCOOH FN), MGP before size fractionation, MGP after size fractionation (fractions 68–73, Fig. 1), and higher molecular weight MGP contaminants (fractions 48–54, Fig. 1). Normal human fibroblasts were added, incubated for 60 min at 37 °C, and adherent cells were quantified by the hexosaminidase assay (see "Experimental Procedures").

FIG. 4. Effect of low molecular weight proteins on cell adhesion to fibronectin. Microtiter plates were coated with 200 μl volumes of the following: 0.1 or 10 μg/ml fibronectin (FN); 5 μg/ml MGP; or with 0.1 μg/ml FN plus 5 μg/ml of either MGP, heat decarboxylated MGP (DeCOOH MGP), cytochrome C (cyt. C), or aprotinin. Normal human fibroblasts were added, incubated for 60 minutes at 37 °C, and adherent cells were quantified by the hexosaminidase assay (see Methods for details). MGP* = MGP after Sephacryl S-200-HR purification.

those used to decarboxylate MGP in the previous study, conditions which abolished the adhesion activity of the MGP preparation (12), the adhesion activity of fibronectin was also in fact inactivated as judged both by the hexosaminidase assay of cell adhesion (Fig. 3) and by the absence of visual evidence of cell attachment or spreading (data not shown). Since fibronectin is not known to contain γ-carboxyglutamic acid residues, it is likely that acid decarboxylation conditions do cause other chemical changes in proteins which can result in loss of biological activity.

Effect of Low Molecular Weight Proteins on the Cell Adhesion Activity of Fibronectin—In the previous study, the possibility that contaminating cell adhesion proteins could account for the cell adhesion activity of the MGP preparation tested was judged to be unlikely because the cell adhesion activity of this MGP preparation was, on a weight basis, even more potent than that of fibronectin. We have confirmed the observation that the cell adhesion activity of MGP preparations prior to size fractionation is greater than that of fibronectin (12). In the experiment shown in Fig. 2, for example, comparable cell adhesion is observed at a coating concentration of the (impure) MGP preparation which is only half of that required for fibronectin. Since the higher molecular weight proteins responsible for this activity account for only 8% of the total protein in the MGP preparation (fractions 48–54, Fig. 1), the actual activity of the higher molecular weight cell adhesion proteins could be 25 times greater than fibronectin on a weight basis. While the as yet unidentified higher molecular weight cell adhesion protein(s) in MGP preparations could be far more potent than fibronectin in the cell adhesion assay, it is also possible that MGP enhances the cell adhesion activity of fibronectin and other cell adhesion molecules. As seen in Fig. 4, the presence of
5 μg of MGP/ml in the coating solution does dramatically enhance the cell adhesion activity observed at 0.1 μg/ml fibronectin, a fibronectin dose which by itself has no cell adhesion activity. Other small proteins also enhance the activity of 0.1 μg/ml fibronectin, including cytochrome c, aprotinin, and heat-decarboxylated MGP (Fig. 4). These results demonstrate that some small molecules can nonspecifically enhance the activity of low doses of fibronectin, an effect which could contribute to the unusually potent cell adhesion activity observed in MGP preparations prior to size fractionation.

**Effect of Purified Anti-bovine MGP Antibodies on the Cell Adhesion Activity of MGP Preparations**—In the previous report that MGP is a cell adhesion protein, evidence was presented that antibodies raised against a synthetic 19-residue peptide corresponding to the C terminus of the 79-residue bovine MGP were unable to block the cell adhesion activity ascribed to MGP (12). This observation was interpreted as evidence that the C terminus of MGP is not involved in the cell adhesion activity of the protein rather than as evidence that MGP itself is not a cell adhesion protein (12). We have carried out two additional antibody tests to explore further the possible role of MGP in cell adhesion. Rabbit antibodies raised against intact bovine MGP were affinity-purified using as adsorbent bovine MGP which had been purified by size fractionation and was devoid of cell adhesion activity. These purified antibodies were unable to alter the dose dependence of the cell adhesion activity obtained with MGP preparations prior to size fractionation when tested at a concentration of 100 μg of antibody/ml. As an additional test, an antibody affinity column was prepared using these affinity-purified anti-bovine MGP antibodies. This column proved to be effective in removing MGP antigen from the MGP preparation prior to size fractionation, as expected, but did not remove the cell adhesion activity.

**DISCUSSION**

The present studies demonstrate that the previously reported cell adhesion activity of MGP (12) is in fact due to higher molecular weight protein contaminants which are present in partially purified MGP preparations. This conclusion accounts for the two apparent anomalies in the previously reported cell adhesion activity of MGP (12), the failure of the antibodies raised against a synthetic peptide corresponding to the C-terminal 19 residues of bovine MGP to inhibit the cell adhesion activity of the bovine protein, and the potent inhibition of the MGP cell adhesion activity by a synthetic peptide containing an RGD sequence, even though MGP does not contain this sequence. Since the cell adhesion activity previously reported in MGP preparations is actually caused by higher molecular weight contaminants, these contaminating cell adhesion molecules cannot be inactivated by antibodies directed against MGP. The contaminating cell adhesion proteins could also contain the RGD sequence, and their adhesion activity would therefore be blocked by synthetic peptides which contain this sequence.

In the course of the present studies, we have developed conditions which can be used to disaggregate MGP so that it can be resolved by size fractionation on a Sephacryl S-200-HR column. Previous studies have shown that MGP strongly self-aggregates to form insoluble precipitates under physiologic conditions (1, 2, 4). Although 8 M urea dissolves MGP, preliminary studies revealed that the elution position of MGP from a Sephacryl S-200-HR column using 8 M urea-containing buffers is far earlier than expected for a protein of 10-kDa size. MGP can also be dissolved in 6 M guanidine HCl and, under the pH 9 conditions described here, MGP elutes from a Sephacryl S-200-HR column at the position expected based on the 10-kDa size of the protein. Since the higher molecular weight contaminants removed from MGP by the gel filtration procedure described here account for 20% of the total protein (Fig. 1) and include potent cell adhesion molecules, this size fractionation procedure should be added to the previously described (16) extraction and ion exchange procedures for the purification of the protein.

**REFERENCES**