Cell Adhesion to Matrix Gla Protein and Its Inhibition by an Arg-Gly-Asp-containing Peptide*

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Matrix Gla protein (MGP) is a 14-kDa protein found in bone and cartilage which contains the unusual amino acid γ-carboxyglutamic acid (Gla). The biological function of this protein has not been elucidated. Here we have demonstrated the adherence of chondrocytes, fibroblasts, osteosarcoma cells, and kidney mesangial cells to MGP purified from bovine bone. Maximum adherence occurred at MGP concentrations of 0.5–1.0 μg/ml. Removal of the calcium-binding Gla residues by thermal decarboxylation of MGP destroyed the proteins' cell adherence properties. Cell adherence to MGP was not affected by the presence of antibodies directed against the C-terminal (non-Gla) portion of the protein or the presence of cycloheximide during the adherence assay. However, the Arg-Gly-Asp-containing synthetic peptide Gly-Arg-Gly-Asp-Ser-Pro significantly inhibited cell attachment to MGP, whereas the control peptide Gly-Arg-Gly-Glu-Ser-Pro had minimal effect. These data indicate that MGP may function in mediating cell attachment to the extracellular matrix via a receptor that requires intact Gla residues and that can be inhibited by Arg-Gly-Asp-containing peptides.

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The abbreviations used are: MGP, matrix Gla protein; Gla, γ-carboxyglutamic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RGD, Arg-Gly-Asp.

The biological function of MGP is not known. Rats treated with warfarin from birth or in utero and humans exposed to warfarin in utero develop growth plate abnormalities that indicate a role for MGP in normal growth plate morphogenesis (5–7). Warfarin treatment results in a disruption of cell migration in the hypertrophic zone of the growth plate (6). Consistent with a role in growth plate development, MGP is expressed at the highest level during the cartilage formation period of endochondral bone development (8). We have recently shown that MGP localizes to the hypertrophic zone of the growth plate and can be found in a cellular and pericellular distribution (9). Due to the location of MGP, its apparent involvement in growth plate development, and the fact that cell adhesion is critical to cell migration and thus morphogenesis, we decided to determine if MGP had cell adherence properties. We found that chondrocytes, fibroblasts, and "osteoblast-like" osteosarcoma cells adhere and spread on MGP-coated surfaces.

EXPERIMENTAL PROCEDURES

Materials—MGP was purified from formic acid extracts of bovine bone as described (9). The purification protocol is very similar to one recently published by Hale et al. (10), which has been shown to result in highly purified MGP. The purified protein was homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was identified as MGP by N-terminal sequence analysis. Decarboxylated MGP was produced as described by Price et al. (11) by acidifying the protein and heating it to 110 °C under vacuum. Bovine fibronectin and the synthetic peptides Gly-Arg-Gly-Asp-Ser-Pro and Gly-Arg-Gly-Glu-Ser-Pro were purchased from Tei- lios Pharmaceuticals (San Diego, CA). Human prothrombin was from Sigma, and bovine serum albumin (BSA) was radioimmunoassay grade and also purchased from Sigma. Microtiter plates used for enzyme-linked immunosorbent assays and for adherence assays were Nunc Immunoplates (Scientific Technologies, Raleigh, NC). Culture media were purchased from Gibco. All chemicals used were analytical reagent-grade or better.

Cell Culture—Bovine articular chondrocytes were cultured as previously described (12). Chondrocytes were not passaged and were used within 2 weeks of the start of culture to assure maintenance of their phenotype. U2-OS cells (human osteosarcoma), HL-60 cells, and HepG2 cells were all obtained from the American Type Culture Collection (Rockville, MD) and were cultured as recommended by the supplier. Rat kidney mesangial cells were a gift from Dr. Richard Appel (Bowman Gray School of Medicine, Winston-Salem, NC) and were cultured as previously described (13). Human dermal fibroblasts were a gift from Dr. Debra Saxe (Emory University School of Medicine, Decatur, GA).

Adherence Assay—Adherence assays were performed on 96-well microtiter plates coated overnight with various substrates. Since MGP is insoluble in aqueous solutions, it was dissolved in 4 M guanidine HCl in 50 mM Tris-HCl, pH 7.6, and then diluted in phosphate-buffered saline (PBS) prior to adsorption to the plates. Adsorption of MGP was verified in preliminary experiments by enzyme-linked immunosorbent assays using MGP antisera previously described (9, 12). Fibronectin, prothrombin, and BSA were diluted in PBS for adsorption. Unbound material was removed and the plates washed three times with PBS. Occupied binding sites were blocked by incubating the plates for 1 h with 3% BSA. Cells were harvested from culture plates with 0.05% trypsin, 0.02% EDTA in PBS and washed twice with 0.02% trypsin inhibitor in PBS and once with serum-free Ham's F-12 medium. Cells were then counted and diluted to 1 x 10⁵ cells/ml. After a 30-min recovery period, 100 μl of serum-free medium and 100 μl of cell suspension were placed in each microtiter well and allowed to incubate at 37 °C for 30–90 min in 5% CO₂ and 100% humidity. Unattached cells were removed and the wells were washed gently three times with PBS. A visual inspection of matrix (4). The biological function of MGP is not known. Rats treated with warfarin from birth or in utero and humans exposed to warfarin in utero develop growth plate abnormalities that indicate a role for MGP in normal growth plate morphogenesis (5–7). Warfarin treatment results in a disruption of cell migration in the hypertrophic zone of the growth plate (6). Consistent with a role in growth plate development, MGP is expressed at the highest level during the cartilage formation period of endochondral bone development (8). We have recently shown that MGP localizes to the hypertrophic zone of the growth plate and can be found in a cellular and pericellular distribution (9). Due to the location of MGP, its apparent involvement in growth plate development, and the fact that cell adhesion is critical to cell migration and thus morphogenesis, we decided to determine if MGP had cell adherence properties. We found that chondrocytes, fibroblasts, and "osteoblast-like" osteosarcoma cells adhere and spread on MGP-coated surfaces.

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of cell attachment was made using an inverted phase-contrast microscope. Bound cells were then quantitated by measuring endogenous hexosaminidase as described (14). Cells were allowed to incubate with the hexosaminidase substrate for 2 h prior to development and measurement of absorbance at 405 nm. In some experiments, cell attachment was quantitated by the release of adherent cells using trypsin/EDTA followed by counting in a Coulter counter. In addition, cell adherence and spreading was visualized by fixing attached cells with 3% paraformaldehyde in PBS and staining with 1% toluidine blue.

**Adherence Inhibition**—Inhibition of adherence by synthetic peptides was studied by incubating the peptide in the incubation medium during the 30-min recovery period prior to the adherence assay and maintaining the peptide in the medium during the adherence assay. To determine the effect of protein synthesis inhibition on adherence, cycloheximide (10 μg/ml) was added to cell cultures for 1–3 h prior to their use in the adherence assay and was present during the assay. Inhibition of adherence by a polyclonal antibody raised against a 19-residue synthetic peptide consisting of the C-terminal sequence of bovine MGP (ERYAMVTGYNAAYDYPRE) previously described (9, 12) was tested by incubating a purified IgG fraction of the antisera in substrate-coated wells for 1 h prior to the adherence assay and maintaining the antibody in the media during the assay.

**RESULTS AND DISCUSSION**

Fig. 1 shows that chondrocytes isolated from bovine articular cartilage attached and spread on microtiter plates coated with 1 μg/ml MGP. Similar adhesion was noted on wells coated with fibronectin (10 μg/ml) but not on wells coated with 3% BSA as negative control. As shown in Fig. 2, maximum attachment of chondrocytes occurred at an MGP concentration of 0.5–1.0 μg/ml. Attachment appeared to decrease at higher concentrations of MGP. A similar finding has been reported with chondrocyte attachment to purified chondronectin and may be due to aggregation of the protein at higher concentrations (15). Attachment of chondrocytes to fibronectin occurred at concentrations of 5–10 μg/ml. As shown in Fig. 2, cells did not attach to prothrombin, indicating that cell attachment to MGP was not the result of nonspecific interactions between the cells and the highly negatively charged Gla residues contained in the vitamin K-dependent proteins. It is unlikely that cell adherence to MGP could have resulted from contamination of the MGP preparation with another cell adherence protein since N-terminal analysis and SDS-PAGE did not reveal any contaminating proteins. The optimal concentration of MGP used in the adherence assays was in the range of 1 μg/ml, which is much lower than the concentration of 10–20 μg/ml necessary for optimal adherence to fibronectin (Fig. 2) and bone phosphoprotein or osteopontin (16–18). To check for the presence of a contaminating cell adherence protein in the BSA (used for blocking) that could potentially bind to MGP and thus mediate adherence, MGP-coated wells were blocked with BSA at concentrations of 3, 1, and 0.5% without any decrease in adherence to MGP (not shown). Also, as shown in Fig. 1, BSA alone did not support any cell adhesion. A previous study by Wong et al. (19) noted cell adherence to a bone matrix protein with the same mobility on SDS-PAGE as MGP. The investigators did not attempt to elucidate the identity of the protein in their study, but we now believe that it was most likely MGP.

Various cell types were tested for their ability to adhere to MGP in comparison to fibronectin and BSA. As shown in Fig. 3, of the cell types tested, chondrocytes and fibroblasts demonstrated the greatest quantitative adhesion to MGP. Adhesion to MGP was also noted to occur with U2-OS cells (human osteosarcoma) and kidney mesangial cells but not with HepG2 (human hepatocellular carcinoma) or HL-60 cells (human leukemia). U2-OS and kidney mesangial cells were noted to adhere more readily to fibronectin (p < 0.01) than to MGP at the concentrations of protein tested. HepG2 and HL-60 cells did not adhere to any of the proteins tested. Visual inspection of cell attachment using an inverted phase-
The integrin family of cell surface proteins is known to facilitate the adhesion of a number of cell types to a variety of extracellular matrix adhesion proteins and Arg-Gly-Asp (RGD)-containing peptides have been shown to inhibit attachment to a number of the integrins (20-22). Therefore, we decided to investigate whether or not adhesion to MGP could also be inhibited by an RGD-containing peptide. As shown in Fig. 4, the synthetic peptide Gly-Arg-Gly-Asp-Ser-Pro, when present during the adherence assay, completely inhibited the adhesion of U2-OS cells to MGP while the control peptide Gly-Arg-Gly-Glu-Ser-Pro had little to no effect on cell adhesion to MGP. The concentration of the RGD peptide necessary to achieve greater than 50% inhibition of adhesion to MGP was in the micromolar range (~5 μM), while similar inhibition of adhesion to fibronectin occurred only when the peptide concentration approached 1 mM. This latter peptide concentration is consistent with previous studies using the same RGD peptide in similar experiments with fibronectin (21). Similar results demonstrating RGD inhibition of adhesion to MGP in the micromolar range were also obtained when chondrocytes and fibroblasts were substituted for U2-OS cells in the adhesion assay (not shown).

The finding that the RGD-containing synthetic peptide inhibited cell adhesion to MGP is intriguing. MGP does not contain an RGD sequence (23). It is possible that cell adhesion to MGP occurs via a receptor that can be occupied by an RGD-containing peptide such as an integrin type receptor. This receptor might recognize a sequence in MGP that presents a conformation and charge similar to that for the RGD sequence. Alternatively, binding to one sequence could affect the conformation of a closely related receptor that recognizes the other sequence. A similar finding has been noted to occur with the glycoprotein Ibα-IIIα complex which recognizes an RGD peptide sequence as well as a completely unrelated sequence, HHLGGAKQAGDY, found in the γ-chain of fibrinogen (24, 25). It appears that the γ-chain fibrinogen peptide and RGD peptides bind to separate subunits that allow “cross-talk” between subunits following occupation by either peptide (24-26).

MGP does not appear to have obvious protein sequence similarity with any of the known cell adhesion recognition sequences reported to date. No obvious similarity was noted in comparison between the bovine MGP sequence (23) and the adhesive recognition sequences of fibronectin, laminin, vitronectin, fibrinogen, von Willebrand factor, entactin, circumsporozoite protein, thrombospondin, collagen type I, collagen type IV, and amyloid P component, recently compiled and reviewed by Yamada (27). In addition, significant sequence similarity was not found between MGP and known cell adhesion proteins by searching the current Swiss-Prot, Protein Information Resource, GenBank, and EMBL data bases. Work is in progress to identify the MGP cell recognition sequence using proteolytic digests of MGP in cell adhesion inhibition assays.

The Gla residues are necessary for the function of the vitamin K-dependent proteins whose functions are known (2). Decarboxylated MGP did not support cell adhesion (Fig. 5). MGP treated in this manner was still capable of adsorbing onto the microtiter plates and was immunoreactive by enzyme-linked immunosorbent assay (not shown). An IgG fraction of polyclonal antiser raised against the 19 C-terminal amino acid sequence (non-Gla region) of MGP did not inhibit cell adhesion to MGP when present during the adherence assay (Fig. 5). In addition, cells did not adhere to wells coated with the MGP C-terminal synthetic peptide (not shown). These findings indicate that the calcium-binding Gla region of MGP is necessary for cell adhesion and the C-terminal portion of MGP probably does not contain the cell recognition site. Adherence to MGP was not dependent upon new protein synthesis during the adherence assay since preincubation of cells for 3 h with cycloheximide at concentrations up to 10 µg/ml did not interfere with the cells’ ability to adhere to MGP (not shown).

In conclusion, we have found that MGP may function as a cell adhesion protein. This is a unique function for one of the members of the vitamin K-dependent protein family. Since MGP appears to associate with other extracellular matrix proteins in bone and cartilage (3), MGP may assist in mediating cell adhesion to one or more of these proteins. An
alteration in cell adhesion to MGP resulting in altered cell migration in the growth plate might explain the skeletal abnormalities noted in animals, including humans, exposed to the vitamin K antagonist warfarin (5-7).

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