Articular-cartilage matrix γ -carboxyglutamic acid-containing protein

Characterization and immunolocalization

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Matrix γ -carboxyglutamic acid (Gla)-containing protein (MGP) was found to be present in articular cartilage by Western-blot analysis of guanidinium chloride extracts of human and bovine cartilage and was further localized by immunohistochemical studies on human and monkey specimens. In newborn articular cartilage MGP was present diffusely throughout the matrix, whereas in growth-plate cartilage it was seen mainly in late hypertrophic and calcifying-zone chondrocytes. In adult articular cartilage MGP was present primarily in chondrocytes and the pericellular matrix. Immunoelectron microscopy studies revealed an association between MGP and vesicular structures with an appearance consistent with matrix vesicles. MGP may be an important regulator of cartilage calcification because of its localization in cartilage and the known affinity of Gla-containing proteins for Ca^{2+} and hydroxyapatite.

INTRODUCTION

Matrix γ -carboxyglutamic acid (Gla)-containing protein (MGP) was initially discovered in the organic matrix of bone and later was found to be present in various cartilages from the rat, including growth plate, costal, tracheal, vertebral disc, xiphoid process, nasal septal and auricular cartilage [1–3]. MGP is characterized as a vitamin K-dependent protein because it contains five Gla residues, which are produced in a post-translational carboxylation reaction that requires reduced vitamin K as cofactor [4]. The Gla residues provide the protein with Ca^{2+} - and hydroxyapatite-binding properties that are important to the protein's proposed role as a regulator of mineralization. Evidence for this role is provided by experiments demonstrating excessive mineralization in growth-plate and nasal-septal cartilage in rats treated with the vitamin K antagonist warfarin, which effectively decreases the amounts of γ -carboxylated vitamin K-dependent proteins [5,6]. A similar disorder of growth-plate mineralization has been noted in children born to mothers treated with warfarin during pregnancy [7].

Because of its potential as a mineralization inhibitor, we are studying MGP in articular cartilage. Abnormal articular-cartilage calcification has been seen in conditions such as chondrocalcinosis, hydroxyapatite deposition and osteoarthritis [8–10] and could be due to a local defect in MGP production. Recently we have discovered that articular-cartilage chondrocytes contain the necessary enzymes for the synthesis of vitamin K-dependent proteins and that they do synthesize MGP [11]. Our present studies demonstrate for the first time the presence and localization of MGP in bovine, monkey and human articular cartilage.

MATERIALS AND METHODS

Extraction and purification of MGP from bone and cartilage

Bone MGP was purified from formic acid extracts of bovine bone as follows. Femurs were obtained from a local slaugh-

terhouse, the epiphyses were removed and the soft tissue was discarded. The bones were cut into chunks, washed, frozen in liquid N_2 and ground in a mill. The resultant bone powder was extracted with a 10-fold excess of 10% (v/v) formic acid in the presence of a proteinase inhibitor mix (5 mM-benzamidine, 10 mM-6-aminohexanoic acid and 30 μM -phenylmethanesulphonyl fluoride) for 2 h at 4 °C. The extract was filtered through a Whatman no. 1 filter to remove undissolved bone particles and then filtered through a Millipore 0.45 μm -pore-size filter. The filtered extract was loaded on to a Pharmacia (Piscataway, NJ, U.S.A.) PepRPC HR 10/10 column, and the bound proteins were eluted with a 0–60% (v/v) acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid. Fractions containing MGP appeared at about 30% (v/v) acetonitrile and were collected and dialysed against 10 mM- NH_4HCO_3 containing 5 mM-benzamidine. A precipitate formed during dialysis, which was collected by centrifugation and dissolved in 8 M-urea/0.1 M-sodium acetate buffer, pH 5.5, and applied to a cation-exchange column (SP-Sephadex C-25; Pharmacia) equilibrated in the same buffer. The column was eluted with a 0–0.75 M-NaCl gradient in the equilibration buffer. A single broad peak of MGP was eluted at approx. 0.5 M-NaCl and was dialysed against 10 mM- NH_4HCO_3 and freeze-dried. The purified protein appeared as a single band with a molecular mass of approx. 14 kDa on a 15% polyacrylamide gel. The protein's identity as MGP was confirmed by *N*-terminal sequence analysis, which agreed with the published sequence [4].

Cartilage MGP was extracted from bovine and human articular cartilage with guanidinium chloride as follows. Bovine cartilage was shaved from fetlock joints as previously described [11]. Human cartilage was obtained from femoral heads at the time of hip replacement surgery for osteoarthritis or femoral neck fracture. Grossly normal appearing cartilage was removed with a scalpel blade, care being taken to avoid underlying bone, and the cartilage was washed in 0.9% NaCl and minced before extraction. Extraction took place overnight at 4 °C with 4 M-guanidinium chloride in 50 mM-sodium acetate buffer, pH 6,

Abbreviations used: Gla, γ -carboxyglutamic acid; MGP, matrix Gla-containing protein.

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containing a proteinase inhibitor mix (0.1 M-6-aminohexanoic acid, 0.1 M-EGTA, 1 mM-benzamidine, 1 mM-di-isopropyl phosphorofluoridate and 10 mM-*N*-ethylmaleimide). The extract was filtered and in some experiments the crude extract was dialysed against 10 mM-NH₄HCO₃ containing 5 mM-benzamidine until a precipitate formed. The precipitate was separated by centrifugation and freeze-dried and represents crude extracts used in some of the experiments described below.

For further purification of MGP from bovine cartilage the filtered extracts were dialysed against 0.32% sodium citrate containing 5 mM-benzamidine and 1 mM-di-isopropyl phosphorofluoridate. A precipitate was separated by centrifugation. Barium citrate was formed in the supernatant by adding 1 M-BaCl₂ and the insoluble salt was obtained by centrifugation. The barium citrate salt was washed three times in 0.9% NaCl before proteins that had adsorbed on the salt were released in 0.2 M-EDTA. The released proteins were dialysed against phosphate-buffered saline (150 mM-NaCl/15 mM-sodium phosphate buffer, pH 7.2) containing 5 mM-benzamidine. Again a precipitate formed and this material was used as a source of purified cartilage MGP in some of the experiments described below.

Preparation of MGP antiserum

Antiserum was obtained by immunizing two rabbits with a peptide whose sequence consisted of the C-terminal 19 amino acid residues of bovine MGP as reported by Price *et al.* [4], Glu-Arg-Tyr-Ala-Met-Val-Tyr-Gly-Tyr-Asn-Ala-Ala-Tyr-Asp-Arg-Tyr-Phe-Arg-Glu, synthesized by Bachem (Torrance, CA, U.S.A.). The peptide was reported by the supplier to be > 98% pure on the basis of their analysis in three different h.p.l.c. systems. The peptide was dissolved in water, and 0.5 mg was emulsified with Freund's complete adjuvant before injection at multiple intradermal sites. A booster injection was made in Freund's incomplete adjuvant after 4 weeks. Serum was withdrawn at regular intervals and tested for immunoreactivity to purified bovine bone MGP in dot-blot and Western-blot assays. The MGP antiserum did not recognize bovine, human or rat osteocalcin (C. M. Gundberg, personal communication). For use in immunohistochemistry, MGP antiserum was affinity-purified on an affinity column constructed by coupling the MGP synthetic peptide to activated CH-Sepharose 4B (Pharmacia) according to the manufacturer's suggestions.

Immunoblotting

SDS/PAGE was performed according to the procedure of Laemmli [12] in 15% gels. Before electrophoresis samples were boiled in 1% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. Standard proteins used for molecular-mass determination were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Transfer of proteins from SDS/PAGE gels to Immobilon-P membranes was performed in a Hoefer TE 42 Transphor Unit (Hoefer Scientific, San Francisco, CA, U.S.A.) in Towbin transfer buffer [25 mM-Tris/192 mM-glycine/20% (v/v) methanol]. After transfer, membranes were blocked for 1 h with 5% (w/v) dried milk powder in 150 mM-NaCl/50 mM-Tris/HCl buffer, pH 7 (Blotto) and then incubated overnight with MGP antiserum diluted 1:500 in Blotto. Immunoreactive protein bands were detected by using horseradish peroxidase-conjugated goat anti-(rabbit IgG) antibodies (Blotting Grade from Bio-Rad Laboratories) diluted 1:3000 in Blotto and detected after reduction with 4-chloro-1-naphthol.

Immunodot blotting was carried out on nitrocellulose membranes with the use of ¹²⁵I-labelled Protein A for quantitative determination of immune complexes as previously described [13].

Immunohistochemistry

Specimens were obtained from the sternal growth plate and femoral condylar articular cartilage of one newborn cynomolgus monkey (*Macaca fascicularis*) and from the femoral condylar articular cartilage, costochondral junction, liver and brain of adult cynomolgus monkeys (7–16 years old). All tissues were fixed in cold 4% paraformaldehyde and embedded (undecalcified) in paraffin. Sections (5 µm thick) of tissue were placed on slides that were pretreated with 2% 3-aminopropyl-triethoxysilane (Sigma Chemical Co.) to improve adhesion of tissue. Tissue sections were heated on the slides for 2 h at 58 °C, allowed to cool and then deparaffinized. Sections were predigested with chondroitin ABC lyase (Sigma Chemical Co.) at 1 unit/ml for 10 min at 37 °C, treated with 3% (v/v) H₂O₂ to quench endogenous peroxidase and blocked with 2% (v/v) normal goat serum. Sections were incubated overnight with affinity-purified primary antibody at 10 µg/ml. Secondary antibody consisted of biotinylated goat anti-(rabbit IgG) antibody (Jackson, West Grove, PA, U.S.A.) at a 1:2000 dilution incubated at room temperature for 30 min. Slides were incubated with streptavidin–horseradish peroxidase complex for 45 min at room temperature followed by 0.06% diaminobenzidine (Sigma Chemical Co.) for 10 min at 37 °C. Slides were washed with phosphate-buffered saline between all appropriate steps above. After colour development, slides were washed in water and counterstained with Harris haematoxylin before mounting. Negative controls consisted of phosphate-buffered saline alone and irrelevant antibodies (anti-macrophage) in place of primary antibody.

Human articular cartilage was obtained from the femoral head of a 74-year-old male at the time of hip replacement surgery for femoral neck fracture. Immunohistochemical staining was performed by using an avidin–biotin detection system with the automatic Fisher Code-On Immunostainer (Fisher Scientific, Pittsburg, PA, U.S.A.) and the Biomedica Autoprobe III Immunohistostystem (Biomedica, Foster City, CA, U.S.A.). Before application of primary antibody, some sections were predigested with 0.2% trypsin or 0.5% hyaluronidase (Sigma Chemical Co.) for 8 min at 45 °C.

Immunoelectron microscopy

Thin slices of femoral articular cartilage (0.5 mm) from an adult cynomolgus monkey were fixed for 1 h in 1% glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.3, containing 0.2 M-sucrose. Fixed tissue was cryoprotected with sucrose infiltration and was then rapidly frozen in liquid Freon at liquid-N₂ temperature and sectioned with a Reichert–Jung cryoultramicrotome. Thin sections for conventional transmission electron microscopy averaged 70 nm in thickness, whereas thicker sections (0.5 µm) were cut for viewing by intermediate voltage electron microscopy.

Immunostaining was performed by using a modification [14] of the Tokuyasu method [15]. Non-specific binding of antibodies was inhibited by incubation in 0.1% glycine in Gey's buffer followed by Gey's buffer containing 2% (w/v) gelatin, 5% (w/v) BSA and 5% (v/v) normal goat serum. Grids were then incubated overnight at 4 °C in either anti-MGP antibody at < 10 µg/ml or rabbit anti-lactoferrin antibody (Cappel, West Chester, PA, U.S.A.) (10 µg/ml) as a control. After incubation with primary antibody, the grids were rinsed in Gey's buffer and incubated in a solution containing goat anti-(rabbit IgG) antibody conjugated to 15 nm gold particles, goat anti-(mouse IgG) antibody conjugated to 5 nm gold particles (Janssen Life Sciences Products, Beerse, Belgium) and Gey's buffer in the proportions 1:2:1. The goat anti-(mouse IgG) antibody–5 nm gold conjugate was used

as an internal control. After incubation in the gold-conjugated second antibodies, the samples were rinsed successively in Gey's buffer and then cacodylate/sucrose buffer and the preparations were fixed with 1% glutaraldehyde in cacodylate/sucrose buffer.

The samples were preserved by using a modification of the ultrathin plastic embedding procedure of Keller *et al.* [16]. Grids were rinsed with buffer, placed on drops of 0.5% osmium tetroxide for 10 min, rinsed on drops of cacodylate buffer, stained *en bloc* with aq. 0.5% uranyl acetate, dehydrated and finally embedded in a drop of LR White resin (London Resin, London, U.K.). Some grids were then post-stained with Reynold's lead citrate.

Thin sections were viewed at 80 keV with a Philips 400 electron microscope, and thicker sections were imaged at 300 keV with the Philips CM-30 intermediate-voltage electron microscope.

RESULTS

MGP was detected in guanidinium chloride extracts of human articular cartilage. SDS/PAGE of a crude protein extract of human articular cartilage and purified bone MGP are shown in Fig. 1, lanes 1 and 2 respectively. Multiple Coomassie Blue-stained protein bands appeared in the crude cartilage extract (Fig. 1a, lane 1) with a band present corresponding to the position of MGP purified from bovine bone (Fig. 1a, lane 2). Samples identical with those in Fig. 1(a) were run concurrently and transferred to an Immobilon-P membrane for Western blotting. As shown in Fig. 1(b), antisera raised against the C-terminal MGP synthetic peptide recognized the purified bone protein (lane 2) and a protein in the cartilage extract (lane 1) at the proper position for MGP. On the basis of densitometric scanning of immunoblots of cartilage extracts and of immunoblots of bone MGP in known concentrations, articular cartilage was estimated to contain 0.5 μ g of MGP/mg of guanidinium chloride-extracted protein.

Fig. 1. SDS/PAGE (a) and Western-blot analysis (b) of purified bone MGP and guanidinium chloride-extracted articular-cartilage proteins

MGP was purified from bovine bone, and proteins from human articular cartilage were extracted with 4 M-guanidinium chloride, as described in the Materials and methods section. The proteins were electrophoresed on a 15% polyacrylamide gel along with unstained (a, lane S) and prestained (b, lane S) molecular-mass standards. Identical samples of bone MGP (a and b, lanes 2) and cartilage extract (a and b, lanes 1) were run on each half of the gel. One half of the gel was stained with Coomassie Brilliant Blue and is shown in (a). The other half of the gel was transferred to an Immobilon-P membrane and processed for Western blotting (b) with antiserum raised against the MGP synthetic peptide as primary antibody.

After transfer to an Immobilon-P membrane, both the bone MGP and the cartilage MGP reacted with 4-diazobenzene-sulphonic acid, which has been shown to detect the presence of Gla in proteins [17], confirming that both the bone MGP and cartilage MGP were γ -carboxylated (results not shown).

Immunolocalization of MGP

At the light-microscopy level, immunostaining of chondrocytes for MGP was noted in articular cartilage taken from the femoral head of a patient undergoing hip replacement for a femoral neck fracture (Fig. 2a). Staining was localized to the cells and the pericellular matrix. Staining for MGP in the remainder of the matrix was minimal and was not enhanced by predigestion of the tissue with either trypsin or hyaluronidase. However, digestion of the specimen with either enzyme did enhance the cellular and pericellular staining. Negative controls did not exhibit staining (Fig. 2b).

Because of the availability of fresh tissue from a variety of sites, further studies were performed with specimens obtained from cynomolgus monkeys. Articular cartilage from the femoral condyle of an adult monkey showed positive immunostaining for MGP in chondrocytes, with cells within 80 μ m of the articular surface staining much more strongly positive than cells further from the articular surface (Fig. 2c). As was noted for the human tissue, most of the staining was concentrated within the cells and pericellular matrix. There was only faint staining of the remainder of the matrix and little or no enhancement of matrix staining with chondroitin lyase predigestion of the sample. Negative controls had no positively stained areas (Fig. 2d). A similar pattern of MGP staining limited mainly to the cells and pericellular matrix was seen in the costochondral cartilage.

In contrast with the adult, femoral condylar articular cartilage from a newborn monkey had very marked and diffuse staining of the matrix (Fig. 2e). Immunostaining for MGP was noted throughout the articular-epiphyseal cartilage complex, including both the superficial region that remains as articular cartilage and the deeper areas that are destined to undergo endochondral ossification [18]. Negative controls did not stain (Fig. 2f). In sternal growth-plate cartilage positive staining for MGP was present in cells in the late hypertrophic and calcifying zones with comparatively little staining in cells in the resting and proliferative zones and little or no matrix staining (Fig. 2g).

As additional controls, studies with the anti-MGP antibody were performed on sections of monkey liver and brain tissue. Fraser & Price [3] have shown that these tissues contain relatively little MGP mRNA and therefore should have little or no staining for MGP if our antibody staining is specific. Indeed, in the liver and brain sections, staining for MGP was noted only in the surrounding connective tissue corresponding to the liver capsule and the meninges of the brain. No staining of the liver (Fig. 2h) or brain parenchyma was noted.

Electron-microscopy studies performed on adult monkey articular cartilage with use of the anti-MGP antibody confirmed the light-microscopic findings and further localized the MGP. MGP was found primarily intracellularly, with relatively little MGP detected in the matrix (Fig. 3a). Cellular staining for MGP was noted in both the cytoplasm and the nucleus. The cytoplasmic MGP appeared to be associated with vesicular or membrane-bound structures (Fig. 3b). Most of the extracellular MGP was found within vesicular structures (Fig. 3c). These extracellular vesicles were the proper size and density for matrix vesicles, measuring on average 0.1 μ m [19]. There was also immunogold staining for MGP in the cell processes. There did not appear to be any clear association of MGP with strands of type II collagen, which could easily be identified by their typical periodicity. Negative controls did not show immunogold staining.

Fig. 2. Immunolocalization of MGP

Immunostaining was performed with affinity-purified anti-MGP antibody ($10 \mu\text{g/ml}$) as described in the Materials and methods section. (a) Human articular cartilage from the femoral head of a 74-year-old male predigested with 0.2% trypsin. Positive (pink) immunostaining for MGP within chondrocytes (bar = $30 \mu\text{m}$). (b) Negative control for (a). The antiserum was pre-absorbed with the immunizing peptide and only blue counterstaining is noted (bar = $30 \mu\text{m}$). (c) Adult monkey articular cartilage predigested with chondroitinase showing positive (brown) immunostaining for MGP in chondrocytes and pericellular matrix most prominent near the articular surface (bar = $100 \mu\text{m}$). (d) Negative control for (c) (bar = $100 \mu\text{m}$). (e) Articular-epiphyseal cartilage complex from the femoral condyle of a newborn monkey predigested with chondroitin lyase. Strong diffuse brown immunostaining of the matrix is seen (bar = $700 \mu\text{m}$). (f) Negative control for (e) (bar = $700 \mu\text{m}$). (g) Sternal growth-plate cartilage from newborn monkey showing strong immunostaining (brown) for MGP in calcifying zone chondrocytes (arrows) (bar = $30 \mu\text{m}$). (h) Section of adult monkey liver immunostained for MGP. Parenchyma is negative but liver capsule (arrow) is positive (bar = $200 \mu\text{m}$).

Fig. 3. Electron micrographs of MGP immunogold labelling of adult monkey articular cartilage

Cryosectioned tissue was incubated with anti-MGP antibody followed by goat anti-rabbit IgG conjugated to 15 nm gold particles as second antibody as described in the Materials and methods section. (a) Immunogold labelling (arrowheads) is denser in the cell cytoplasm than in the matrix (bar = 1 μm). (b) High-magnification electron micrograph of a portion of a chondrocyte demonstrating immunogold staining of intracellular vesicles (arrowheads) (bar = 0.5 μm). (c) Interterritorial matrix demonstrating lack of preferential immunogold staining of collagen, whereas dense immunogold labelling of vesicles (arrowheads) is noted (bar = 0.5 μm).

DISCUSSION

This is the first demonstration that a vitamin K-dependent protein, MGP, is present in adult articular cartilage obtained from primates including humans. Previous studies [2,3] had shown that MGP was present in various types of rat cartilage; however, articular cartilage was not examined. MGP appears to represent about 0.05% of the total amount of cartilage protein extracted with guanidinium chloride, and the concentration of 0.5 $\mu\text{g}/\text{mg}$ of tissue protein is consistent with concentrations noted in rat cartilages [2]. It has been shown that articular-cartilage chondrocytes are capable of synthesizing a family of vitamin K-dependent proteins including MGP [11]. MGP is the first of this family to be isolated and characterized. The presence of MGP in articular cartilage and the fact that vitamin K is necessary for synthesis of the carboxylated protein indicate that vitamin K may be important in normal cartilage function and

that the use of vitamin K antagonists such as warfarin may have an effect on adult articular cartilage, as has been noted for growth-plate cartilage [5,7].

In addition to cartilage, MGP also has been demonstrated to be present in both the mineral and the organic matrix of bone, from which the protein was initially isolated and purified [1]. We chose to purify MGP from formic acid extracts of bone since, as noted by Otawara & Price [20], over half of the MGP is removed from adult bone during demineralization with formic acid. It has been suggested that cartilage MGP from the rat is identical with rat bone MGP [2]. Our results reveal that bone and cartilage MGP migrate similarly on SDS/PAGE and both react with 4-diazobenzenesulphonic acid, indicating the presence of Gla in cartilage and bone MGP.

The immunolocalization studies indicate that, at least in the adult, MGP is primarily a cell-associated protein. Staining for MGP was concentrated within the chondrocytes with some

staining in the pericellular matrix but little to no staining in the territorial or interterritorial matrices. It would be possible for the antigen to be present in the matrix with its epitope blocked and unavailable for reaction with the antibody, but digestion of the sections with trypsin, hyaluronidase or chondroitin lyase did not significantly enhance the matrix staining, although it did enhance the cellular and pericellular staining somewhat. It is unlikely that MGP was lost from the interterritorial matrix during preparation of the samples since pretreatment with enzyme digestion enhanced rather than diminished the cellular and pericellular staining and since staining was noted in the extracellular matrix of cartilage samples from a newborn animal prepared in the same way as the adult samples.

A significant difference from the adult specimens was noted in cartilage obtained from a newborn animal. In articular cartilage from the newborn monkey, there was marked diffuse matrix staining for MGP in the territorial and interterritorial matrices. This would indicate that either much greater amounts of MGP are present in newborn articular cartilage as compared with the adult or that the MGP in the cartilage matrix from the newborn animal is more accessible to the MGP antibody because of other matrix differences between young and adult animals. In support of the former, studies of rat bone [20] showed that a much greater proportion of MGP was extracted from the organic matrix of newborn compared with adult bone.

The immunostaining noted in the present studies appears to be due to specific binding of antibody to MGP. The antibody was affinity-purified because of the known problem of non-specific binding of polyclonal rabbit serum to cartilage [21]. The antibody only reacted with a protein at the appropriate position for MGP and not with other proteins in a crude guanidinium chloride extract of articular cartilage. Appropriate immunochemistry controls were negative, as were liver and brain tissue, where MGP should not be present in significant amounts. In the study by Fraser & Price [3], samples of liver and brain had only very small amounts of MGP mRNA. In the present study, staining for MGP was limited to the surrounding connective tissue and was not present in the parenchyma, indicating that the MGP message noted in these tissues by Fraser & Price may have come from small amounts of connective tissue in the brain and liver samples.

In sections of growth-plate cartilage the cells closest to or within areas of mineralization, i.e. the hypertrophic and calcified cartilage zone cells, stained more heavily for MGP than did the more superficial cells. Interestingly, it has recently been shown that the hypertrophic zone of growth plate is also the area most affected in the rat fetus with warfarin administration [22]. This is consistent with the notion that MGP is involved in the regulation of mineralization and with the known affinity of Gla-containing proteins for hydroxyapatite. The deeply positive staining of MGP in the articular-epiphyseal cartilage complex in a newborn monkey also supports this theory, as all but the thin articular cartilage region of this tissue will undergo endochondral ossification. However, as it has been previously noted [2,3], MGP is not found exclusively in areas where mineralization occurs but also appears in soft tissues that do not normally mineralize. This tissue distribution of MGP could indicate that MGP either functions as an inhibitor of mineralization as proposed previously [3], or has a different function in mineralized versus non-mineralized tissue, or has a function independent of mineralization.

The demonstration by electron microscopy of an association between MGP and vesicles in the chondrocytes and matrix indicates that this protein is present in matrix vesicles. Matrix

vesicles have been thought by some to be involved in the initiation of epiphyseal cartilage mineralization [19,23,24]. However, they have been noted in normal non-calcifying articular cartilage and in articular cartilage from patients with osteoarthritis [10,25]. Although their exact origin is unclear, they may represent broken-off cell processes or lipid debris from dying cells [25]. If matrix vesicles or, in the case of pathological mineralization, cellular lipid debris are involved in mineralization, then the presence of MGP in these structures could indeed support a role for MGP in the regulation of cartilage mineralization.

In conclusion, a vitamin K-dependent Gla-containing protein, MGP, is clearly present in articular cartilage. In the adult, it is found primarily in a cellular and pericellular location. MGP is also present in areas of cartilage destined for mineralization including the articular-epiphyseal cartilage complex and the hypertrophic and calcified cartilage zone of growth-plate cartilage as well as in matrix vesicles. Therefore MGP may play an important role in the regulation of cartilage mineralization. Further studies are needed to clarify this role.

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REFERENCES

- Price, P. A., Urist, M. R. & Otawara, Y. (1983) *Biochem. Biophys. Res. Commun.* **117**, 765-771
- Hale, J. E., Fraser, J. D. & Price, P. A. (1988) *J. Biol. Chem.* **263**, 5820-5824
- Fraser, J. D. & Price, P. A. (1988) *J. Biol. Chem.* **263**, 11033-11036
- Price, P. A. & Williamson, M. K. (1985) *J. Biol. Chem.* **260**, 14971-14975
- Price, P. A., Williamson, M. K., Haba, T., Dell, R. B. & Jee, W. S. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7734-7738
- Price, P. A. (1989) *Connect. Tissue Res.* **21**, 51-60
- Hall, J. G., Pauli, R. M. & Wilson, K. M. (1980) *Am. J. Med.* **68**, 122-140
- Gordon, G. V., Villanueva, T., Schumacher, H. R. & Gohel, V. (1984) *J. Rheumatol.* **11**, 681-686
- Doyle, D. V. (1982) *J. Pathol.* **136**, 199-216
- Ali, S. Y. (1985) *Scanning Electron Microsc.* **4**, 1555-1566
- Loeser, R. & Wallin, R. (1991) *Connect. Tissue Res.* **26**, 135-144
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685
- Wallin, R. & Martin, L. F. (1988) *J. Biol. Chem.* **263**, 9994-10001
- Willard, A. S., Lewis, J. C., Thomas, M. J. & Taylor, R. G. (1990) *Proc. Int. Congr. Electron Microsc.* 12th **12**, 364-365
- Tokuyasu, K. T. (1980) *Histochem. J.* **12**, 381-403
- Keller G., Tokuyasu, K. T., Dutton, A. H. & Singer, S. J. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5744
- Nasimoto, S. K. (1990) *Anal. Biochem.* **186**, 273-279
- Carlson, C. S., Hilley, H. D. & Henrickson, C. K. (1985) *Am. J. Vet. Res.* **46**, 306-313
- Ali, S. Y., Sajdera, S. W. & Anderson, H. C. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 1513-1520
- Otawara, Y. & Price, P. A. (1986) *J. Biol. Chem.* **261**, 10828-10832
- Heinegård, D. & Paulsson, M. (1987) *Methods Enzymol.* **145D**, 336-363
- Feteih, R., Tassinari, M. S. & Lian, J. B. (1990) *J. Bone Miner. Res.* **5**, 885-894
- Anderson, H. C. (1969) *J. Cell Biol.* **41**, 59
- Poole, A. R., Matsui, Y., Hinek, A. & Lee, E. R. (1989) *Anat. Rec.* **224**, 167-179
- Mitchell, N. S. & Shepard, N. L. (1990) *Anat. Rec.* **227**, 397-404