Basic fibroblast growth factor as a selective inducer of matrix Gla protein gene expression in proliferative chondrocytes

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Matrix Gla protein (MGP) is a member of the vitamin K-dependent γ carboxylase protein family expressed in cartilage. Insulin-like growth factor I (IGF1) stimulates chondrocyte differentiation, whereas basic fibroblast growth factor (FGF2) acts in an opposite manner. We explored the differential expression and regulation by IGF1 and FGF2 of the MGP gene during chondrocyte differentiation. We used a primary culture system of rabbit epiphyseal chondrocytes to show that MGP mRNA is mainly expressed during serum-induced proliferation. Much lower MGP mRNA content is observed in post-mitotic chondrocytes, which newly express α1X procollagen mRNA, a marker of late-differentiated cells. From studies of a series of growth factors, it was shown that IGF1 decreased chondrocyte MGP transcripts, whereas FGF2 had the opposite effect. FGF2 stimulated chondrocyte MGP production in a dose- and time-dependent manner at the mRNA and protein levels. FGF2 acted in a dose- and time-dependent manner, reaching a maximum at 10 ng/ml at 20 h. The protein synthesis inhibitor cycloheximide did not modify FGF2 action, in agreement with a direct effect. Actinomycin D abolished FGF2-induced stimulation, strongly suggesting that FGF2 modulated MGP gene transcription. We transiently transfected chondrocytes with a construct containing the mouse MGP promoter from -5000 to -168 base pairs, relative to the transcription start site of the gene linked to the luciferase gene (MGP-Luc). In transfected cells, FGF2 stimulated luciferase activity up to sevenfold while IGF1 had no effect. Hence, FGF2 induces transcription of the MGP gene via the 5′-flanking region of the gene. Using a series of deleted MGP-Luc constructs, we identified a sequence of 748 base pairs which was sufficient for transcriptional activation by FGF2. These results led us to postulate that the inhibitory chondrogenic action of FGF2 involves a mechanism whereby MGP gene transcription and protein are induced.

Key words: growth plate cartilage, growth factors, skeletal growth.

INTRODUCTION

During endochondral ossification, the chondrocytes present in the early cartilaginous pattern of skeletal bones, and later in the growth plate, progress through a complex differentiation process that involves proliferation and terminal differentiation (hypertrophy and apoptosis). Once fully differentiated, hypertrophic chondrocytes undergo cell death and participate in the mineralization of the cartilaginous matrix which is progressively replaced by bone. This leads to longitudinal bone growth. Extracellular matrix proteins, growth factors and hormones play important roles in regulating the differentiation and maturation process of chondrocytes.

Among the extracellular matrix proteins produced by chondrocytes, the matrix Gla protein (MGP), which belongs to the vitamin K-dependent γ carboxylase protein family, was proposed as a new marker of chondrogenesis [1]. MGP was initially extracted and purified from demineralized bone powder [2]. However, in skeleton, MGP is exclusively produced by chondrocytes [3], whereas osteocalcin, another Gla protein, is present in bone [4]. In theory, the structural properties of MGP Gla residues should promote binding to calcium and phosphate ions. Hence, the hypothesis arose that MGP could serve as a regulator of mineralization. Indeed, excessive mineralization was observed in growth plate cartilage in rats treated with the vitamin K antagonist warfarin [5,6], and inappropriate calcification of cartilage was reported in MGP-deficient mice [7]. Recent data demonstrated that mutations in MGP are responsible for Keutel syndrome, an autosomal recessive disorder partly characterized by abnormal cartilage calcification [8]. Taken together these genetic data demonstrate that MGP is an inhibitor of cartilage matrix calcification.

Studies of MGP developmental expression in skeletal tissues showed that this protein was produced very early into the cartilaginous pattern of long bones prior to its replacement by bone tissue [1,9]. However, MGP mRNA expression is not exclusively located in the calcifying zone of cartilage, but it is also expressed at other stages of chondrocyte maturation, during embryonic development as well as after birth. During mouse development, MGP mRNA was observed by *in situ* hybridization in late hypertrophic chondrocytes of growth plate cartilage and also in the resting and proliferative chondrocytes which do not mineralize [1]. After birth, MGP was immunolocalized in the calcifying zone of growth plate cartilage of new born monkey, and also in adult bovine and human articular cartilage [10]. The stage-specific biphasic expression of MGP was also observed *in vitro* in the ATDC5 mouse chondrogenic cell line [11].

Little information is available about the factors controlling MGP gene expression in cartilage or other tissues and cells expressing MGP. It was shown that retinoic acid increases MGP mRNA expression in some cell lines (human fibroblasts, chondrocytes, osteoblasts, osteosarcoma cell line MG-63 and rat type II

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; FGF2, basic fibroblast growth factor; GAPDH, glyceraldehyde phosphate dehydrogenase; GH, growth hormone; IGF1, insulin-like growth factor I; MGP, matrix Gla protein; PDGF, platelet-derived growth factor; SSC, standard sodium citrate; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

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pneumocytes), although it decreased it in others (human breast cancer cells MCF-7 and rat kidney cells NRK52E) [12,13]. Transforming growth factor (TGF) β increased MGP mRNA expression in cultured embryonic lung cells [14] and basic fibroblast growth factor (FGF2) dramatically reduced it in rat kidney cells [12].

In the present work we addressed two different but complementary issues. First, we studied the pattern of MGP gene expression in chondrocytes which present different stages of differentiation *in vitro*. We used a high density culture system in which rabbit epiphyseal chondrocytes progressed through a differentiation pathway, including proliferation and post-mitotic steps. We show that MGP is strongly expressed in serum- and FGF2-stimulated proliferating chondrocytes, whereas it is at the limit of detection in late-differentiated chondrocytes which express type X collagen. Secondly, we investigated the mechanism by which FGF2 regulated MGP gene expression. We present evidence showing that FGF2 induces MGP via a transcriptional mechanism involving a specific region of the gene.

MATERIALS AND METHODS

Rabbit cartilage cell culture

Prepubertal (15- to 30-day-old) female *Fauve de Bourgogne* rabbits (Centre production animale, Olivet, France) were killed according to the French legislation of animals. Muscles, aponeurosis and periosteum were removed from long bones. Chondrocytes were released from cartilage by sequential enzymic digestion, and cultured as previouly described [15]. Briefly, cartilage was cut into small pieces and digested at 37 °C successively with 0.05 % hyaluronidase (Roche–Boehringer Mannheim) in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL–Life Technologies) for 10 min, 0.2 % trypsin (Roche–Boehringer Mannheim) for 30 min, then with 0.2 % collagenase (Roche–Boehringer Mannheim) for 60 min. Cells were plated at a density of 1.2×10^4 cells/cm² in 75-cm² culture flasks, each containing 12 ml of Ham's F12 medium (Sigma Chemical Co.) plus 10 % foetal calf serum (FCS), 10 units/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical Co). Cells were incubated at 37 °C in a 10 % CO_2 atmosphere, allowing them to adhere to the culture flask. Medium was changed every three days during the first 21 days, after which it was replaced with DMEM containing 2 % FCS for an additional period of 8 days. At successive periods of culture, chondrocytes were incubated in serum-free DMEM with or without one of the following human recombinant growth factors: basic fibroblast growth factor (FGF2 or bFGF; Roche–Boehringer Mannheim), growth hormone (GH; Novo Nordisk A/S, Bagsværd Danmark), insulin-like growth factor 1 (IGF1; Upstate Biotechnology, Lake Placid, NY, U.S.A.), platelet-derived growth factor (PDGF; Upstate Biotechnology), TGFβ1 (R & D Systems) or vascular endothelial growth factor (VEGF; Upstate Biotechnology) at a concentration varying between 1 and 100 ng/ml. The medium was removed 24 h later and total RNA was extracted from the cell pellet.

In some experiments, pieces of rabbit skin (instead of cartilage tissue) was submitted to a similar sequential digestion. The resulting fibroblast suspension was then cultured as described above, and RNA extraction was performed when the cells reached confluency.

Culture of vascular cells

The following vascular cells from different species and different origins were used in this study: mouse endothelial cell line b.End3 (ECACC ref. no. 96091929), rat smooth musle cell line A 7r5 (kindly provided by A. Gadeau, INSERM U 441, Bordeaux, France), and rat vascular smooth musle cells in primary culture (a gift from J. B. Michel, INSERM U 460, Paris, France). All cell types were maintained in exponential growth in Ham's F12 medium supplemented with 10 % FCS at 37° in a 10 % CO_2 atmosphere. Rabbit vascular smooth musle cell line (U8A4) was a gift from J. M. Daniel-Lamazière (INSERM U 441, Bordeaux, France). These cells were cultured at 33 °C in a medium containing 50 % Ham's F12, 50 % DMEM, 1 g/l glucose, 5μg/ml transferrin (Sigma Chemical Co.), 6 ng/ml sodium selenite (Sigma Chemical Co.), and 200 nM ascorbate (Merck). Cells were then incubated in serum-free DMEM with or without recombinant human GH, IGF1, PDGF, TGFβ1, VEGF or FGF2, each at a concentration between 1 and 100 ng/ml. Medium was removed after 24 h and the cells were harvested and total RNA was extracted from the cell pellet.

Northern blot analysis of total RNA

Total cellular RNA was extracted from different rabbit tissues or from cultured cells. Cartilage, lung, aorta, kidney, liver, skin and striated muscles were dissected in sterile conditions and rapidly frozen and powdered in liquid nitrogen. Total RNA extracts were recovered according to Chomczynsky and Sacchi [16] and quantified by measuring the absorbance at 260 nm. RNA samples (8 μg) were electrophoresed in RNA denaturing 2.2 M formaldehyde/1 % agarose gels. RNA was transferred on to a GeneScreen nylon membrane (NEN Life Science, Boston, MA, U.S.A.) and was immobilized by baking the membrane at 80 °C for 20 min and UV crosslinking. Prehybridization and hybridization were performed with the Rapid-Hyb buffer kit (Amersham Life Sciences) according to the manufacturer's protocol.

The following cDNA probes were used: a 223 bp MGP cDNA containing the coding region for exons 2 to 4 of the mouse MGP gene [1]; a rabbit MGP cDNA probe synthesized by reverse transcription and PCR amplification using the following oligonucleotide primers: 5′-CTGCCACCCACCCAGAGGAC-3′ (position 27) and 5′-GGGGGCAAGAAGGAAGGGTG-3′ (position 492) (EMBL D21235); the cDNA encoding the C propeptide region of the bovine pro α1 II procollagen [17]; the 1.8 kb cDNA-encoded part of the triple helical domain of the human collagen α1 I chain [18]; and a 650 bp fragment of pSAM10 (gift from Dr Mallein-Gerin, CNRS UPR 412, France) encoding the NC1-coding domain and the 3′-untranslated domain of the human procollagen α1X [19]. Human glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA was used as an internal control. Probes were labelled by random priming with [α-^{32}P]dCTP (Amersham) to a mean specific activity of 10^8 to 10^9 c.p.m./μg. The hybridized membranes were washed with 0.1 % SDS in 2 × standard sodium citrate (SSC) buffer [1 × SSC buffer is 3M sodium chloride, 0.3M trisodium citrate (pH 7.0)] at 20–22 °C for 20 min, with 0.1 % SDS in 1 × SSC at 65 °C for 10 min, and then exposed to Cronex film at −80 ° for 16 h. Quantitative results were performed using scanning densitometry. Cross-reactivity of the mouse MGP probe with rabbit tissues was evaluated and found to be positive (results not shown).

Immunological study

Chondrocytes were cultured in 4-chamber Labtek slides (Merck) and incubated for 8 h in serum-free DMEM, with or without FGF2 at 5 or 20 ng/ml. After being washed in PBS, cells were fixed in cold methanol at −20° for 6 min, then washed in

blocking buffer (PBS containing 5% BSA and 0.2% Tween 20). Fixed cells were then exposed to the rabbit polyclonal MGP antibody, raised to a peptide corresponding to the C-terminal region of mouse MGP [11]. Antibody was diluted at 1/200 in blocking buffer, and incubation with cells was carried out for 105 min at 20–22 °C in a humid chamber. After a quick rinse in 0.2% Tween 20 in PBS, the goat anti-rabbit FITC-coupled secondary antibody (Transduction Laboratories, Lexington, KY, U.S.A.) diluted at 1/100 in blocking buffer was deposited on the slides for 45 min, followed by a final rinse in 0.2% Tween 20 in PBS. Slides were then mounted with Vectashield, containing 4,6-diamidino-2-phenylindole ('DAPI') (Vector Lab, Burlingame, CA, U.S.A.), examined with a Nikon Labophot 2 equipped with an FITC filter and photographed with Spot Jr (Diagnostic Instruments, Sterling Heights, MI, U.S.A.). Immunofluorescence controls were made by omission of primary antibody.

Western blotting

Proteins were extracted from the cultured cells by addition of SDS buffer [31 mM Tris/HCl (pH 6.8) containing 1% SDS, 5% (v/v) glycerol, 0.01% Bromophenol Blue and 1% 2-mercaptoethanol] [20] and size-separated by SDS/PAGE in a 15% polyacrylamide gel. Coloured Rainbow mix markers (Amersham) were used as molecular size standards. The gel was electroblotted on to nitrocellulose (Bio-Rad) in transfer buffer [25 mM Tris/HCl (pH 8.3), 192 mM glycine] in Transblot cells (Bio-Rad). Equal transfer of proteins was confirmed by staining the nitrocellulose with 0.2% Ponceau Red (w/v) diluted 99:1 in 10% trifluoroacetic acid. The MGP antibody diluted at 1/200 was used as a primary antibody and incubated overnight at 4 °C. A peroxidase-conjugated goat anti-rabbit IgG (Sigma Chemical Co.) was used as secondary antibody at 1/2000 and incubated for 1 h at 20–20 °C. Blots were revealed by enhanced chemiluminescence as instructed by the manufacturer (Amersham).

DNA constructions

The promoter of rabbit MGP has not yet been determined. Since the rabbit, human and mouse MGP mRNA have approx. 80% identity, we used pMGP1 (256 bp), pMGP2 (816 bp) and pMGP4 (5068 bp) fragments of the mouse MGP promoter inserted into the pA4luc vector, as described by Luo et al. [1]. The plasmid pMGP3 was a 1084 bp fragment of the mouse MGP gene that was amplified by PCR using the following primers: 5′-CCGCTCGAGGTGCTTCTGAGACCCAAGAC-3′ (position -1016) and 5′-CCCAAGCTTGTGAGTCTGCCTCTGTGTCT-3′ (position +68), so that the amplified product could be directionally inserted between the *Xho*I and *Hin*dIII sites of the pA4luc vector, as described by Wood et al. [21]. The orientation and sequence fidelity of the cloned fragments was confirmed using a 373A DNA Sequencer (Perkin Elmer).

Transient transfection and luciferase assay

Epiphyseal chondrocytes were grown in Ham's F12 with 10% FCS as described above. On days 15 to 18, during the proliferative phase of culture, cells were trypsinized and plated in P60 culture dishes. Cells were transfected at 70% confluence using calcium phosphate precipitation. Briefly, cells were incubated for 4 h with the precipitate obtained with 2.5 M $CaCl_2$ in a Hepes buffer solution (pH 6.95) containing 1.5 M Na_2HPO_4, 280 mM NaCl and DNA. The DNA content was either 8 μg of pMGP1, pMGP2, pMGP3 or pMGP4 with 2 μg of pRSVβ gal, a plasmid used as an internal control which contains the *Escherichia coli* lac Z gene fused to the long terminal repeat promoter of the Rous sarcoma virus. Cells were then subjected to a 15% (v/v) glycerol shock for 90 min and incubated in DMEM containing 10% (v/v) FCS for 20 h. The medium was replaced with serum-free DMEM alone or serum-free DMEM containing each effector, and cells were further incubated for an additional 20 h. Cells were then lysed in 400 μl of lysis buffer (Promega). Samples (10 μl) of each cell extract were used in triplicate for determination of protein content, luciferase and β-galactosidase activities. Data were expressed as luciferase activity normalized to the β-galactosidase activity. The β-galactosidase activity was evaluated with the β-galactosidase enzyme assay system (Promega).

Similar experiments were carried out using NIH/3T3 fibroblasts (ATCC CCL-92) as negative controls.

DNA content

The DNA content of cultured chondrocytes was measured by the fluorimetric method of Kapuscinski and Skoczylas [22].

Statistical analysis

Student's t test was used for statistical analysis. P values above 0.05 were not considered statistically significant.

RESULTS

In vivo and *in vitro* MGP mRNA content in cartilage tissue and cells from post-natal rabbits

The MGP mRNA content of post-natal rabbit epiphyseal growth plate cartilage was evaluated and compared with that of other tissues, particularly blood vessels containing smooth musle cells. As shown in Figure 1, a single MGP transcript at 0.7 kb is

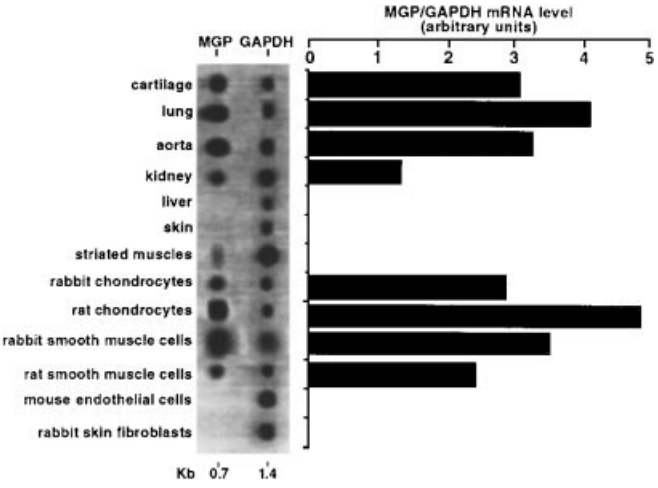

Figure 1 Northern blot analysis showing the expression of MGP mRNA in rabbit tissues *in vivo* and in various cell types *in vitro* from different species

Total RNA was extracted either from prepubertal rabbit epipheseal cartilage, lung, liver, skin, kidney, striated muscles and blood vessels or from different cell types: rabbit and rat epiphyseal chondrocytes, rabbit skin fibroblasts, rabbit (U8A4) and rat (A7r5) vascular smooth muscle cells and mouse endothelial cells (b.End3). The left-hand side of the Figure shows the hybridization signal to the mouse MGP probe (16 h exposure) and to a human GAPDH probe (4 h exposure), indicating the relative amount of RNA loaded in each lane. The right-hand side shows the densitometric quantification of the autoradiogram expressed as the MGP/GAPDH band density ratio. Data shown are representative of three independent experiments.

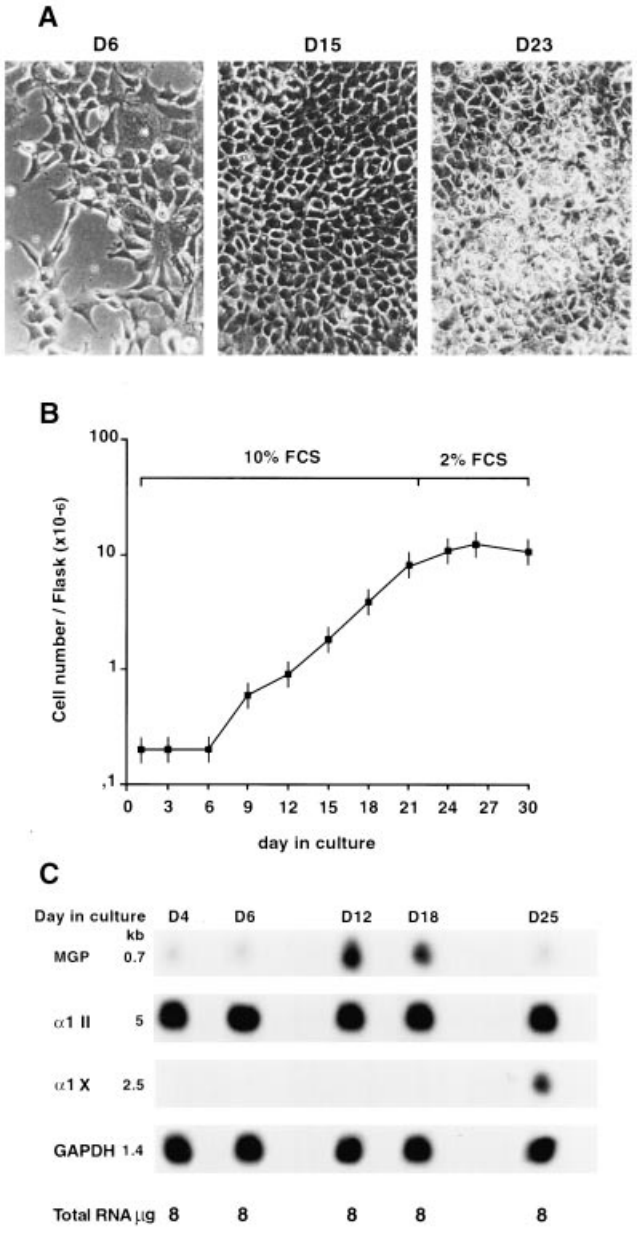

Figure 2 Rabbit epipheseal chondrocytes at successive stages of primary culture

(**A**) Morphology of rabbit epipheseal chondrocytes at three successive periods of primary culture. Days (D) 6, 12 and 23 are shown. (**B**) Cell number measurements were performed at successive stages of primary culture, during cell proliferation (days 6 to 24) and after cells stopped dividing (days 24 to 30). (**C**) Northern blot showing MGP, α1 II procollagen (α1 II) and α1 X procollagen (α1 X) hybridization signals. The amount of RNA loaded in each lane was monitored by hybridization with GAPDH cDNA. Data shown are representative of six independent experiments.

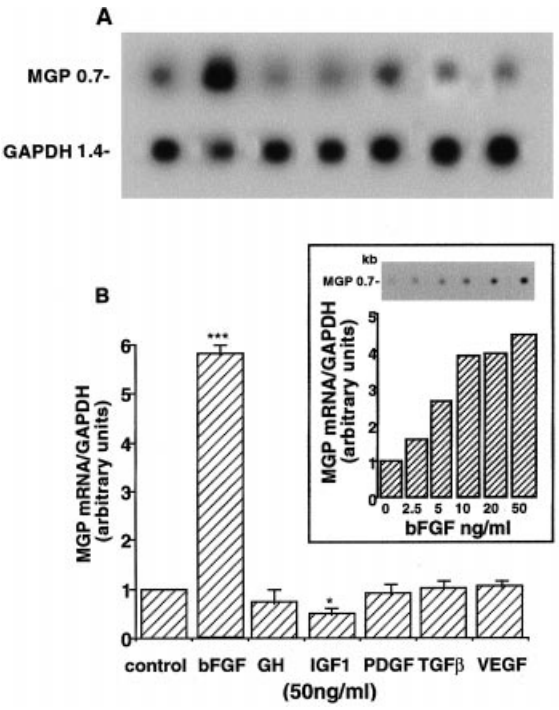

Figure 3 Effect of growth factors on MGP mRNA in proliferative epiphyseal chondrocytes

(**A**) Northern blot showing the effect of different growth factors on MGP mRNA in proliferative epiphyseal chondrocytes. Chondrocytes were cultured for 12 days and then transferred into serum-free DMEM with 50 ng/ml of either FGF2, GH, IGF1, PDGF, TGFβ or VEGF for 24 h. Total RNA was extracted, as described in the Materials and methods section. (**B**) Densitometric quantification of the autoradiogram is expressed as the ratio of MGP mRNA to the corresponding GAPDH mRNA. Data from treated cells are compared with that of control cells, incubated in the absence of any added compound, and referred to as one unit. Results represent the mean $\pm$ S.D. of data obtained from three independent experiments. Dose responsiveness of the MGP mRNA content of chondrocytes to FGF2 is shown in the inset.

expressed at similarly strong levels in cartilage, lung (an organ rich in cartilage bronchi) and aorta, whereas a weaker expression is found in kidney. MGP mRNA was below the limit of detection in liver, skin and striated muscles.

At the cellular level, MGP mRNA is observed in primary cultures of chondrocytes from rabbit and rat epiphyseal cartilage (Figure 1). MGP mRNA is also detected in rat and rabbit vascular smooth muscle cell lines, whereas it was below the level of detection in rabbit skin fibroblasts and mouse endothelial cells.

MGP mRNA expression in epiphyseal chondrocytes in primary culture

To examine the variation of MGP mRNA expression during chondrocyte maturation, chondrocytes were isolated from prepubertal rabbit epiphyseal growth plate cartilage and grown in primary culture for 30 days (Figure 2A). Cells incubated in DMEM containing 10 % FCS did not proliferate during the first 6 days (Figure 2A, D6). The mean DNA content remained at 10 ± 0.8 μg per flask and the cells grew slowly and formed multilayered colonies up to day 24 (Figure 2A, D15–D23). Between days 9 and 21, the doubling time was between 2.5 and 3 days (Figure 2B). On day 21, the cells were incubated with DMEM containing 2 % FCS for an additional period of 9 days, during which they stopped dividing. The DNA content reached a plateau at 440 ± 55 μg per flask. At days 4, 6, 12, 18 and 25, total RNA was extracted and analysed for variations in MGP, α1 II procollagen and α1X procollagen mRNAs by Northern blot (Figure 2C). The GAPDH mRNA was used as a control. The α1 II procollagen mRNA, the specific marker of chondrocyte, was expressed at a similar steady-state level during the whole culture process. In this high density culture system, in which chondrocytes develop as multilayered colonies, no significant expression

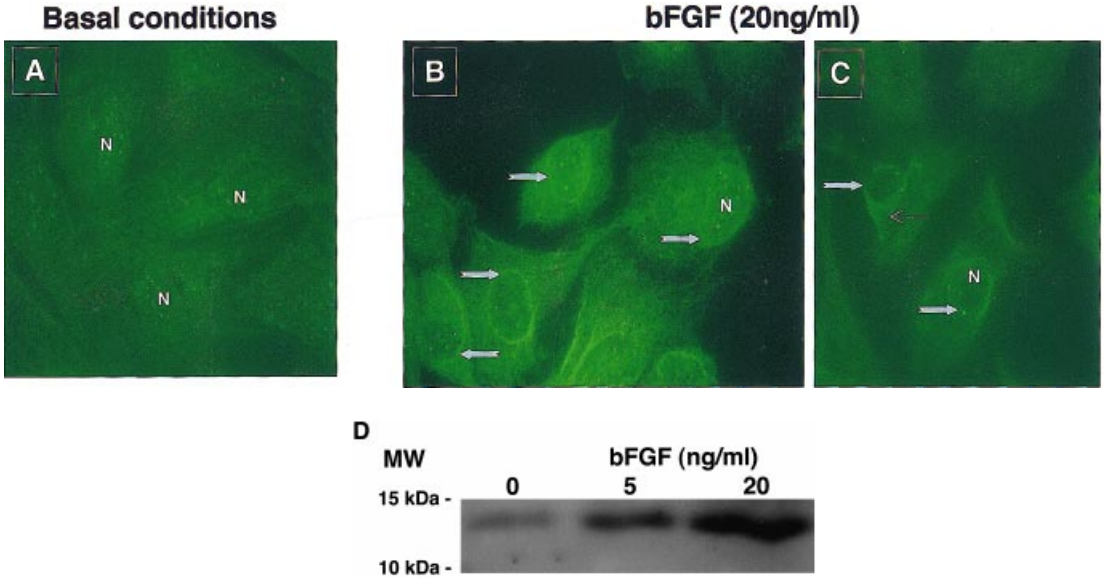

Figure 4 Immunocytochemistry and Western blotting of the FGF2-stimulated expression of MGP

Chondrocytes were incubated for 8 h in serum-free DMEM alone (basal conditions) or serum-free DMEM containing FGF2 at 5 or 20 ng/ml. In one series of experiments, cells were fixed with methanol and incubated with anti-mouse MGP antiserum. Immunocytochemistry of chondrocytes incubated in basal conditions (**A**) and in the presence of 20 ng/ml FGF2 (bFGF) (**B**,**C**). N, nucleus; plain arrows, perinuclear overstaining; dotted arrow, Golgi area overstaining; magnification $\times 100$. In another set of experiments, Western blot analysis confirmed the expression of MGP as a single band identified between 10 and 15 kDa (**D**). The intensity of the MGP band observed in chondrocytes treated with 5 or 20 ng/ml bFGF was compared with that observed in basal conditions. Equal loading of protein was confirmed by Ponceau Red staining. Molecular size markers are indicated on the left.

of α1 I procollagen mRNA was detected (results not shown). By contrast, the α1X procollagen mRNA was only present within the late days of culture (Figure 2C, D25).

The levels of MGP mRNA varied with the stage of culture. It was at the limit of detection at day 4 and day 6, then markedly higher expression at day 12 and day 18 during the proliferation phase, and became nearly undetectable at day 25 when α1X procollagen mRNA was present (Figure 2C). Hence, MGP mRNA concentration is positively correlated with the proliferation of chondrocytes cultured in the presence of 10 % FCS.

Effect of growth factors

Next we sought to determine whether MGP expression could be modulated by mitogenic factors. To address this question, we cultured epiphyseal chondrocytes as described above for 12 days, then for an additional 24 h in serum-free DMEM alone or containing FGF2, GH, IGF1, PDGF, TGFβ1 or VEGF at 50 ng/ml. Figure 3(A) shows a representative Northern blot probed with MGP and with GAPDH as a control. Among the effectors, only FGF2 up-regulated MGP mRNA (about 6-fold higher than the control, Figure 3B). The stimulating effect was dose-dependent, reaching a maximum at 10 ng/ml and a plateau thereafter (Figure 3B, inset). In contrast to the FGF2 induction, IGF1 decreased levels of MGP mRNA by 50 % (Figure 3B). None of the other tested effectors affected MGP mRNA expression, whatever the concentration (1–100 ng/ml) (results not shown).

Analysis of FGF2 action on MGP gene expression

We first wondered whether FGF2 induction of MGP mRNA correlated with an increase in MGP protein. Chondrocytes on day 12 of culture were incubated in serum-free DMEM with FGF2 at 5 or 20 ng/ml or serum-free DMEM alone (control) for 8 h. Immunocytochemistry experiments indicated that FGF2 induced staining in the perinuclear area, which was stronger in almost all cells when compared with the staining in controls (compare Figures 4B and 4C with Figure 4A). In some FGF2-treated cells, strong MGP staining is seen in the area of the Golgi apparatus (Figure 4C, dotted arrow). The observed overstained perinuclear immunoreactivity supports the idea that FGF2 induces newly synthesized MGP, which is located in the ribosomal circular area of the cells. Western blot analysis of chondrocyte proteins with MGP antiserum shows that the band between 10 and 15 kDa is stimulated 2.5- and 6-fold in the presence of 5 and 10 ng/ml FGF2 respectively (Figure 4D). Altogether, these data indicate that FGF2 induces MGP protein in chondrocytes.

We then asked the question whether FGF2 action on the MGP gene was direct or indirect. To assess this, we incubated cells with the protein inhibitor cycloheximide for 18 h, with or without FGF2, and analysed MGP mRNA content. As shown in Figure 5, cycloheximide slightly reduced basal MGP mRNA but did not affect FGF2 induction. This indicated that ongoing protein synthesis was not required for the effect of FGF2 to occur.

The third question was whether FGF2 induction of MGP mRNA was exerted at the level of MGP gene transcription. Cells were treated with 5 μg/ml actinomycin D to block transcription, then exposed to 20 ng/ml FGF2 for 18 h. Actinomycin D had no effect on MGP mRNA in untreated cells, but abolished FGF2-induced increase in MGP mRNA (Figure 5).

Finally, we carried out a series of experiments showing that FGF2 did not modify MGP mRNA turnover (results not shown). Altogether, these results, combined with the demonstration that the mechanism is direct, strongly suggest that FGF2 modulates MGP gene transcription.

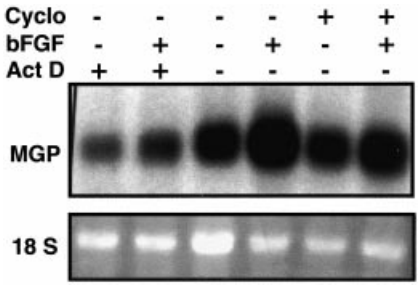

Figure 5 Influence of actinomycin D and cycloheximide on the FGF2 modulation of chondrocyte MGP mRNA

Chondrocytes were cultured for 12 days and then transferred into serum-free DMEM with or without either 5 μg/ml actinomycine D (Act D) or 1.5 μg/ml cycloheximide (Cyclo) for 30 min. Cells were then treated with 20 ng/ml FGF2 (bFGF) for 24 h before RNA extraction and analysis by Northern blot. Control cells were not treated with FGF2. A typical autoradiogram obtained after hybridization with the MGP probe (performed as described in the Materials and methods section) is shown. The corresponding ethidium bromide staining of the 18S rRNA signal was used to normalize MGP mRNA levels. The autoradiogram is representative of two independent experiments.

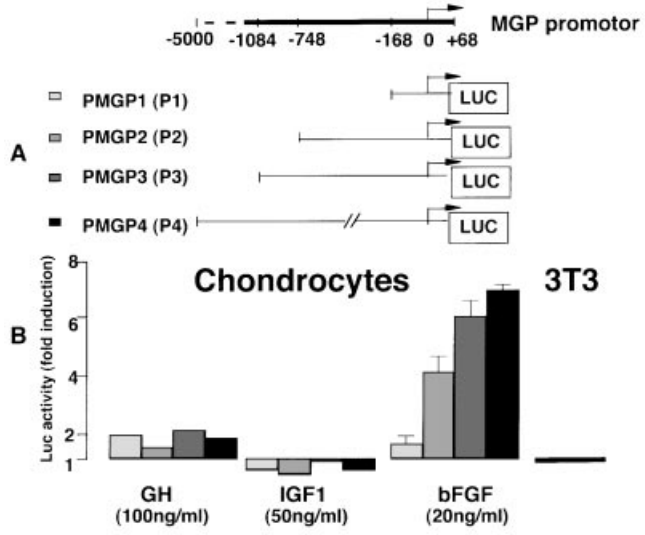

Figure 6 MGP promoter activity

The effects of human GH, IGF1 or FGF2 on the expression of different fragments of the mouse MGP gene promoter. (**A**) The design of the four constructs used in DNA transfection experiments is shown. Each expression vector represents a fragment of MGP promoter linked to the luciferase (LUC) reporter gene. (**B**) Relative luciferase activity. Fragments of MGP gene promoter were co-transfected with a β-galactosidase expression vector into proliferative chondrocytes and treated with each effector for 24 h. 3T3 cells similarly transfected were used as negative controls. For each construct, the luciferase (Luc) activity was calculated as 'fold induction' in FGF2 (bFGF) treated cells with respect to the basal activity measured in untreated cells. Values for luciferase activity are normalized to the β galactosidase activity. Results are mean $\pm$ S.D. of ten independent experiments.

Localization of the FGF2 response region in the MGP gene promoter

To further analyse FGF2 mechanism of action, we transiently transfected chondrocytes with MGP promoter constructs to determine whether the promoter region of the MGP gene was the target of FGF2 action. For that purpose we made four plasmid constructs in which -5000 bp (pMGP4), -1084 bp (pMGP3), -748 bp (pMGP2) or -168 bp (pMGP1) of the 5′-flanking region of the mouse MGP gene was linked to the luciferase gene. Proliferative chondrocytes were transiently co-transfected with either one of the constructs or pA4Luc, in which the SV40 promoter was linked to the lacZ gene (used as a standard for transfection efficiency). Following a 24 h recovery period after transfection, cells were deprived of FCS and further incubated for 24 h with FGF2, GH or IGF1. None of the MGP promoter fragments tested responded to GH or IGF1. In contrast, FGF2 strongly stimulated luciferase activity in cells transfected with pMGP4 (7-fold), pMGP3 (6-fold) and pMGP2 (4-fold) (Figure 6). In contrast, pMGP1-transfected cells did not respond. Hence, the FGF2 response region is between -168 and -748 bp of the MGP gene promoter. The stimulating effect of FGF2 on pMGP4 luciferase activity is dose-dependent, with a maximum effect reached at 20 ng/ml (results not shown). As expected, the four chimaeric genes were unable to drive luciferase reporter gene expression in 3T3 fibroblasts, confirming that MGP gene expression is cell-type specific (Figure 6).

DISCUSSION

In the present work we show that MGP is mainly expressed in serum-induced proliferative chondrocytes, whereas it is weaker in serum-deprived cells and in differentiated chondrocytes expressing type X collagen. Moreover, we demonstrate that IGF1 and FGF2, which have opposing effects on chondrocyte maturation *in vivo*, have the reverse effects on MGP expression: while IGF1 decreases MGP mRNA, our results strongly suggest that FGF2 stimulates MGP gene transcription.

Tissue-specific MGP expression in post-natal immature rabbits is similar to that observed in rats [23]. The presence of MGP transcript that we observe in rabbit aorta probably reflects MGP expression in smooth muscle cells. Our results, obtained using rabbit and rat vascular smooth muscle cell lines in culture, as well as those by Shanahan et al. [24] and Wallin et al. [25], show that these cells express MGP mRNA, whereas mouse endothelial cells do not. Similar findings were also reported by others who found high levels of MGP mRNA in human [26,27] and bovine [28] vascular smooth muscle cells.

We used a high density culture system in which epiphyseal chondrocytes progress through a differentiation pathway modulated by different concentrations of FCS in the culture medium. Arrest of cell proliferation and activation of type X collagen expression by chondrocytes is observed when the concentration of FCS is reduced from 10 % to 2 %. In this system, chondrocytes do not enter into the late hypertrophic stage observed *in vivo* which is characterized by the calcification process of the matrix. We observe that mRNA expression is stronger in serum-induced proliferative chondrocytes than in post-mitotic cells. Moreover, there is an inverse relationship between type X collagen and MGP mRNA expression in non-dividing chondrocytes. These *in vitro* data are in accordance with those reported by Luo et al. [1], who showed that in adjacent sections of growth plate cartilage tissue *in vivo*, MGP expression is turned off in hypertrophic chondrocytes when these cells strongly express the type X collagen gene. These data are also in accordance with the biphasic expression of MGP in the differentiated chondrocytes of the ATDC5 cell line [11].

Since MGP is expressed in proliferative chondrocytes, we asked whether the stimulating effect of serum could be mediated and/or modulated by growth factors. We studied the effect of several growth factors with mitogenic activity, among which IGF1 and FGF2 were shown to be specifically involved in chondrocyte proliferation and maturation, each having opposing effects. IGF1 mediates the stimulating effect of GH on longi-

tudinal bone growth *in vivo*, by increasing terminal differentiation in growth plate chondrocytes [29,30]. In IGF1-null mice, chondrocyte proliferation is normal but the area of hypertrophic chondrocytes is reduced in a linear dimension [31]. FGF2, a member of the fibroblast growth factor family [32], is implicated in mouse chondrogenesis during the earliest stages of limb development [33], and also during post-natal skeletal growth [34]. Its effects are mediated by a family of tyrosine kinase cell surface receptors encoded by four different genes. In humans, mutations in FGFR3 are associated with skeletal disorders [35]. Disruption of the murine FGFR3 gene results in expansion of proliferating and hypertrophic chondrocytes within the growth plate cartilage [33]. *In vitro*, FGF2 has been shown to stabilize the phenotype of chondrocytes plated at low density and to inhibit differentiation into hypertrophic chondrocytes [36,37].

In the present work, we show that FGF2 increases the expression of chondrocyte MGP mRNA, whereas IGF1 has the opposite effect. These results suggest that the control of chondrocyte maturation by growth factors could be, at least in part, mediated by the local production of MGP. Such a hypothesis is in accordance with the work of Yagami et al. [38], who showed that MGP overexpression in chick developing limb delayed chondrocyte maturation and blocked endochondral ossification. More recently, Newman et al. [11] used the ATD5 mouse chondrogenic cell line which allows the *in vitro* study of chondrocyte gene expression during normal endochondral ossification. These authors showed that, depending on the stage of chondrocyte differentiation, over- or under-expression of MGP had different effects on chondrocyte survival and/or maturation. This could explain why, in MGP-null mice, the lack of the MGP gene has different effects on cartilage and arteries depending on the age of the animals. In 4-week-old MGP-null mice, proliferative chondrocytes of the growth plate cartilage were not organized in columns and hypertrophic chondrocytes were absent, whereas in older animals, analysis of affected arteries showed the existence of cartilaginous metaplasia, marked by the presence of chondrocyte-like cells, including hypertrophic chondrocytes [7]. Further studies are needed to better understand the tissue or cell specificity of MGP gene expression.

Our results are the first to report that FGF2: (i) does increase MGP mRNA expression at the transcriptional level; (ii) is acting via a DNA sequence present between -800 bp and -168 bp relative to the transcription start site of the MGP gene, which is sufficient for transcriptional activation; and (iii) exerts a cell-specific action since it is not observed in NIH 3T3 fibroblasts. In the mouse MGP promoter, binding sites for AP1 and AP2 elements can be found, along with potential binding sites for cAMP-dependent transcription factors. However, the transcriptional activation of chondrocyte MGP promoter by FGF2 is independent of the presence of cycloheximide, suggesting that this effect does not require ongoing protein synthesis. The FGF2 effect on target cells is complex. The main pathway is mediated by the membrane FGF receptor, involving dimerization, autophosphorylation and consequently the activation of multiple signalling proteins and second messengers [39]. However, there is evidence to suggest that these receptors may not be sufficient for all aspects of FGF2 signalling [40,41]. Five isoforms of FGF2 have been characterized. The low-molecular-mass isoform (18 kDa) is responsible for the paracrine–autocrine effects of FGF2, and the four nuclear high-molecular-mass isoforms are responsible for the intracrine effects [42,43,44]. FGF2 is internalized through two pathways: a high affinity receptor-mediated pathway and a low affinity receptor-mediated pathway. *In vitro*, in cells grown in the presence of FGF2, all the isoforms are internalized. Some of the FGF2 is recovered into the nuclear fraction, probably regulating directly or indirectly transcription of specific genes [1]. Thus it can be suggested that FGF2 acts directly on the MGP promoter as a transcription factor.

In conclusion, this work presents new evidence that, besides the negative control of cartilage mineralization, MGP could modulate the chondrocyte maturation process. Our data also strongly suggest that FGF2 could be implicated in the latter phenomenon. The mechanisms by which MGP acts at the protein level remain to be determined.

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