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Coordinated expression of matrix Gla protein is required during endochondral ossification for chondrocyte survival

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Matrix Gla protein (MGP) is a 14-kD extracellular matrix protein of the mineral-binding Gla protein family. Studies of MGP-deficient mice suggest that MGP is an inhibitor of extracellular matrix calcification in arteries and the epiphyseal growth plate. In the mammalian growth plate, MGP is expressed by proliferative and late hypertrophic chondrocytes, but not by the intervening chondrocytes. To investigate the functional significance of this biphasic expression pattern, we used the ATDC5 mouse chondrogenic cell line. We found that after induction of the cell line with insulin, the differentiating chondrocytes express MGP in a stage-specific biphasic manner as in vivo. Treatment of the ATDC5 cultures with MGP antiserum during the proliferative phase leads to their apoptosis before maturation, whereas treatment during the hypertrophic phase has no effect on chondrocyte viability or mineralization. After stable transfection of ATDC5 cells with inducible sense or antisense MGP cDNA constructs, we found that overexpression of MGP in maturing chondrocytes and underexpression of MGP in proliferative and hypertrophic chondrocytes induced apoptosis. However, overexpression of MGP during the hypertrophic phase has no effect on chondrocyte viability, but it does reduce mineralization. This work suggests that coordinated levels of MGP are required for chondrocyte differentiation and matrix mineralization.

Introduction

Matrix Gla protein (MGP)* is a 14-kD member of the mineral-binding Gla protein family (Price and Williamson, 1985), which includes a number of coagulation factors, osteocalcin, and the growth arrest–specific protein 6 (Gas6). MGP binds calcium ions and hydroxyapatite via its five γ-carboxylated glutamic acid (Gla) residues. The MGP gene is widely expressed, but the protein only accumulates significantly in bone, cartilage, and dentin (Hale et al., 1988). Within the mammalian growth plate during endochondral bone formation, MGP gene expression is confined to the proliferative and late hypertrophic chondrocytes, but is absent in the maturing/early hypertrophic chondrocytes (Luo et al., 1995).

The MGP-deficient mouse is characterized by aberrant arterial and cartilage calcification, providing compelling evidence that MGP is an important inhibitor of extracellular matrix mineralization in these tissues (Luo et al., 1997). Further, inhibition of the γ-carboxylation of Gla residues with warfarin in both cell culture and in vivo results in increased matrix mineralization, suggesting that the mineral-binding Gla residues of proteins such as MGP are crucial for the regulation of matrix mineralization (Price et al., 1998; Yagami et al., 1999). In addition, the rare autosomal recessive condition Keutel syndrome, in which patients are affected by aberrant cartilage calcification, has been shown to be due to mutations in the gene encoding MGP (Munroe et al., 1999).

As well as a role in matrix calcification, it has been proposed that MGP may have additional functions. Evidence in support of this hypothesis includes the arteries in the MGP-deficient mouse undergoing chondrocyte metaplasia, which indicates that MGP may have a role in cellular differentiation (Luo et al., 1997). Further, the epiphyseal growth plate is disorganized in the MGP-deficient mouse (Luo et al., 1997), in that the proliferative chondrocytes fail to form regular palisaded columns and hypertrophic chondrocytes are absent. In this situation, it is unclear whether the aberrant calcification causes disruption of the normal proliferative chondrocyte zone architecture, or if MGP has a direct influence on chondrocyte maturation. However, misexpression of MGP in chick embryo limb buds has been shown to in-

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*Abbreviations used in this paper: BMP, bone morphogenetic protein; Gas6, growth arrest specific protein 6; MGP, matrix Gla protein; RT, reverse transcriptase; VSMC, vascular smooth muscle cells.

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hibit the formation of hypertrophic chondrocytes during endochondral bone growth (Yagami et al., 1999). Altered MGP gene expression has also been demonstrated in a variety of metastatic cancers and tumor cell lines (Chen et al., 1990; Levedakou et al., 1992; Clark et al., 2000).

To explore the functions of MGP during chondrocyte differentiation, we have used the mouse chondrogenic cell line ATDC5. This cell line has been extensively characterized and shown to be an elegant model to study gene expression and phenotypic changes during normal endochondral ossification (Atsumi et al., 1990; Shukunami et al., 1996, 1997). With insulin supplementation, the ATDC5 cells rapidly proliferate to form a confluent cell monolayer. After 7 d of culture, the cells form discrete condensations and express type II collagen (Atsumi et al., 1990). The condensations differentiate to form cartilage nodules and type X collagen, a marker of chondrocyte hypertrophy (Reichenberger et al., 1991), is expressed by day 15. At day 21, the medium is supplemented with ascorbic acid and the carbon dioxide levels are reduced to encourage calcification of the matrix in the absence of β-glycerophosphate via a matrix vesicle–mediated mechanism. This is evident from day 30 onwards (Shukunami et al., 1997). This cell culture system has enabled us to examine the effects of both over- and underexpression of MGP at specific stages of chondrocyte differentiation. Our findings have identified that coordinately regulated levels of MGP during chondrocyte differentiation are crucial for chondrocyte survival. In addition, we have confirmed a role for MGP in regulating mineralization of hypertrophic chondrocytes.

Results

Gene expression and characterization of ATDC5 cells

The ATDC5 cells proliferated rapidly in insulin-supplemented medium after plating out and attained confluence by day 5. Condensation of cells was evident by day 9 and cartilage nodule formation by day 15. After alteration of culture conditions, calcification of the matrix could be detected by day 32, and by day 42 a significant proportion of the culture was stained positively with alizarin red. These morphological changes are consistent with the stepwise differentiation described previously (Atsumi et al., 1990; Shukunami et al., 1997).

Gene expression at various time points was assessed by northern analysis to compare patterns with previous studies and to determine the expression pattern of MGP (Fig. 1). Expression was analyzed at five time points during ATDC5 differentiation: confluent cultures (day 5), chondrocyte condensation (day 9), cartilage nodule formation (day 15), evidence of chondrocyte hypertrophy (day 21), and matrix mineralization (day 32). We identified expression of type II collagen from day 9 onwards, concomitant with the chondrocytes becoming elongated and forming recognizable cellular condensations. Type X collagen gene expression was detectable at day 15 and increased at day 21 as the chondrocytes were maturing to hypertrophy. MGP expression was detectable at low levels at day 9, but significantly present at day 15. These results are comparable with those of previous studies (Shukunami et al., 1996; Thomas et al., 2000). The level of MGP was lower at day 21, but increased again at day 32. This mirrors the biphasic (on-off-on) expression pattern described in the growth plate (Luo et al., 1995). Expression of the osteoblast-specific protein, osteocalcin, was detectable at day 32, consistent with previous work (Akiyama et al., 1999) suggesting that the ATDC5 cells attain an osteoblastic phenotype.

Treatment of ATDC5 cultures with MGP antisera

To establish the effects of manipulating MGP in ATDC5 cells, we treated cultures with an antiserum to MGP. We confirmed the specificity of the antiserum raised to a peptide corresponding to the COOH-terminal region of mouse MGP by both Western analysis (Fig. 2) and immunohistochemical analysis of bovine fetal growth plates, which showed staining of proliferative and hypertrophic chondrocytes, but not the intervening maturing chondrocytes (results not shown). The ATDC5 cells were treated with the antiserum from either day 3 or from day 21 and at each subsequent medium change. The cells treated with the antiserum from day 3 began to condense and grew normally until day 10, when the cells detached from the culture dishes (Fig. 3), whereas control cultures treated with normal rabbit serum differentiated normally through to matrix mineralization. To determine whether the cells in the antibody-treated cultures were dying by apoptosis, we trypsinized the adherent cells and fixed them in formaldehyde before staining with Hoescht 33258. Analysis under ultraviolet light revealed that the MGP antiserum–treated chondrocytes had typical apoptotic morphology with nuclei that were fragmented and condensed (Fig. 4). The chondrocytes treated with rabbit serum showed no alteration. To confirm these findings, we established the expression levels of *bcl-2* in both the antiserum-treated and control ATDC5 cells. Bcl-2 is a member of a family of apoptosis regulatory gene products and specifically acts as an antagonist of the effector stage of apoptosis (Kroemer, 1997). In the antiserum-treated cultures, levels of expression of *bcl-2* were significantly higher ($P < 0.02$) than in the control cultures at the time point immediately before chondrocyte detachment (Fig. 5). In addition, the *bcl-2:bax* ratio was threefold greater in the control cultures compared with the antiserum-treated cultures at the same time point. This suggests that the antiserum-treated chondrocytes were dying due to apoptosis. Real time PCR analysis of type II collagen revealed no significant difference between antiserum-treated and control cultures (Fig. 5), supporting the specific effect of the antiserum and confirming that the cultures were similarly differentiated. In addition, levels of MGP transcripts were decreased in the antiserum-related cultures just before chondrocyte apoptosis (Fig. 5). This suggests that one of the effects of the antiserum interaction with MGP was to lower transcription of MGP at this stage of chondrocyte differentiation. MGP transcription is potentially influenced in a feedback mechanism dependent on the MGP in the chondrocytes' environment.

The cells treated with the antiserum from day 21 grew normally until day 42 and there was no statistical difference in the degree of matrix mineralization in the treated and control cultures by measurement of calcium levels ($P = 0.59$; Fig. 6).

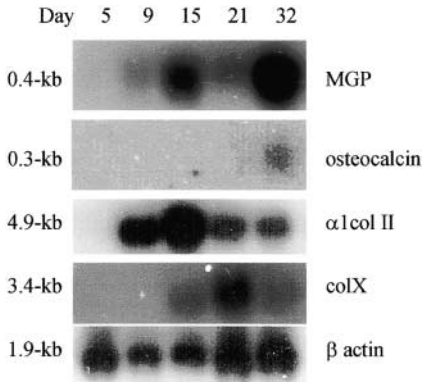

Figure 1. **Expression of cartilage and bone markers during ATDC5 cell differentiation.** Northern blot analysis was performed on 20 μg of total RNA extracted from ATDC5 cell cultures on day 5 (confluence), day 9 (condensation), day 15 (nodule formation), day 21 (maturation), and day 32 (chondrocyte hypertrophy and matrix mineralization). Filters were serially hybridized with murine cDNA probes for type II collagen, type X collagen, osteocalcin, MGP, and β-actin. Transcript sizes are indicated on the left.

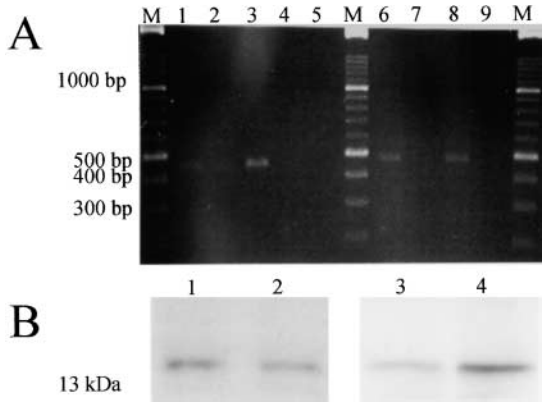

Figure 2. **Confirmation of inducible expression by RT-PCR and Western blotting.** (A) Detection of inducible cDNA transcripts by RT-PCR from ATDC5 cultures transfected with pINDE/X (sense construct) or transfected with pINDE/B (antisense construct, AS-MGP) and induced with ponasterone A from day 3 of culture or noninduced as a control. Total RNA was extracted at day 7 of cell culture and the RT-cDNA was amplified by PCR for 23 cycles. Aliquots (5 μl) of the PCR products were resolved on 1.5% (wt/vol) agarose gels alongside molecular weight markers (M). Lane 1, clone E/X 3 induced; lane 2, clone E/X3 noninduced; lane 3, clone E/X 5 induced; lane 4, clone E/X5 noninduced; lane 5, control; lane 6, clone E/B2 induced; lane 7, clone E/B2 noninduced; lane 8, clone E/B6 induced; lane 9, clone E/B6 noninduced. The primers used for the target sequences were the ecdysone forward primer and mgpR for pINDE/X (to generate a 450-bp fragment and the ecdysone forward primer), and mgpF for pINDE/B (to generate a 471-bp fragment). (B) Western blot analysis confirming the specificity of the MGP antiserum. A single band is identified at ~14 kD. In addition, different levels of MGP are identified in the transfected compared with control cultures. Lane 1, high intensity band corresponding to MGP in chondrocytes with overexpressed MGP compared with lane 2 (control). Lane 3, low intensity band at 14 kD corresponding to MGP in sample from cells with induced AS-MGP compared with lane 4 (control). Equal loading of protein and efficiency of transfer was confirmed by Ponceau S staining. Molecular weight marker sizes are indicated on the left.

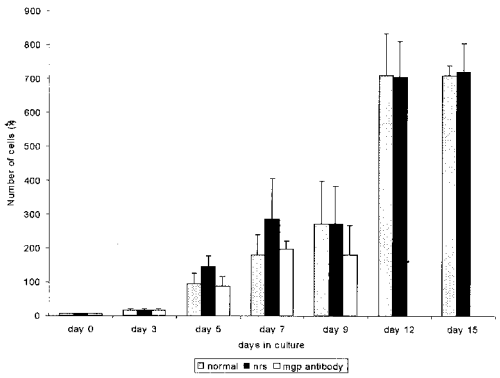

Figure 3. **Cell proliferation of ATDC5 cells treated with MGP antiserum.** Cell proliferation of ATDC5 cells from day 0–15. Cultures were treated with (a) standard culture conditions (normal), (b) normal rabbit serum (nrs) at 1:200 dilution, or (c) antiserum raised to a mouse MGP peptide (1:200). Cells supplemented with MGP antiserum apoptosed and detached on day 10.

Inducible inhibition of MGP expression by antisense transcripts in ATDC5 cells

To determine the effects of decreased levels of MGP at various stages of chondrocyte differentiation, we adopted an antisense RNA approach. We constructed an ecdysone-inducible plasmid (pIND) which contained a 416-bp fragment of mouse MGP cDNA subcloned into the EcoR1 and BamH1 sites of the pIND multiple cloning site in the antisense (3′ to 5′) orientation under the transcriptional control of the ecdysone responsive promoter. After dual transfection of ATDC5 cells with the pIND antisense construct (pINDE/B) and pVgRXR, cells were selected in G418 and Zeocin-containing medium for 14 d. Six stably transfected clones were picked by ring cloning and expanded.

Clone E/B2, which expressed the inducible antisense MGP cDNA, was selected and plated out in multiple 6-well plates. From day 3, 5 μM ponasterone A was added to the medium at each medium change. After induction with ponasterone A, total RNA was extracted from the induced and

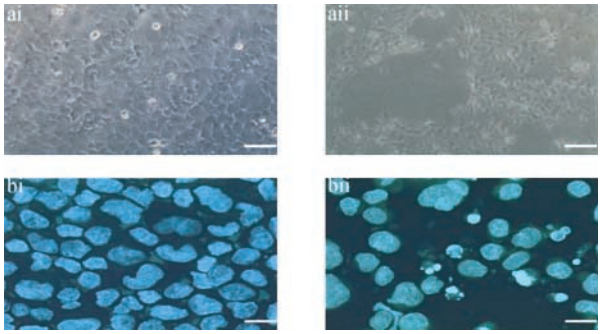

Figure 4. **Effects of MGP antiserum on ATDC5 cells.** Phase-contrast microscopy of ATDC5 cells treated with MGP antiserum after 10 d of culture. (ai) Control culture treated with normal rabbit serum (1:200) from day 3. (aii) Cultures treated with MGP antiserum (1:200) from day 3. Cells were stained with Hoescht 33258 to determine nuclear morphology of (bi) control culture treated with normal rabbit serum and (bii) cultures treated with MGP antiserum showing nuclear condensation and fragmentation consistent with apoptosis. Bars: (ai and aii) 120 μm; (bi and bii) 10 μm.

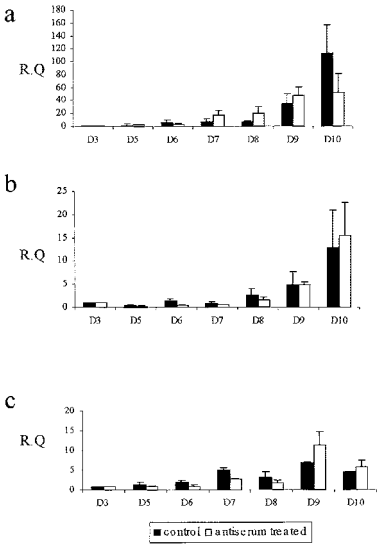

Figure 5. **Real time PCR analysis of MGP antiserum–treated ATDC5 cells.** Real time PCR analysis of levels of (a) MGP transcripts, (b) type II collagen transcripts, and (c) Bcl-2 transcripts in ATDC5 cells treated with MGP antiserum or control cultures (treated with normal rabbit serum) at different time points. R.Q indicates relative quantitation. The expression levels were normalized to β-actin and expressed relative to the levels at day 3, when the antiserum was initially added to the cultures. Note lower levels of MGP and higher levels of Bcl-2 in the antiserum-treated cultures before chondrocyte death. The results are shown as the mean and SD of four independent determinations.

noninduced expanded clones and RT-PCR was performed to confirm inducible expression of the antisense construct (Fig. 2). Real time PCR analysis confirmed that MGP levels were reduced in the induced, transfected cells to ~20% compared with the noninduced cells. The effect of the antisense transcripts was confirmed at the protein level by Western blot analysis (Fig. 2).

The induced transfected cells underwent condensation and proliferated normally until day 11 (Fig. 7), when they changed morphology and detached. The detached cells exhibited typical apoptotic morphology, and their DNA was cleaved into internucleosomal fragments, confirming that the cell death was by apoptosis (precisely as observed for the MGP antiserum–treated cultures; Fig. 4). Noninduced, transfected cell control cultures continued to grow normally. These results show that suppression of MGP expression at an early stage of chondrogenesis does not prevent condensation, but that once cells have condensed the chondrocytes are unable to mature further to form cartilage nodules.

Expanded E/B2 cells were also induced from day 12 to 26 and day 21 to 26 with ponasterone A to assess the effects of inhibiting MGP synthesis on ATDC5 cells during their proliferative, maturing, and hypertrophic phases. In both experiments, the chondrocytes grew normally and cartilage nodules formed until day 26, at which stage the cells apoptosed and detached (results not shown). In contrast, the noninduced cultures grew to 42 d, when normal levels of matrix mineralization were seen. These findings suggest that MGP is vital in the hypertrophic phase of ATDC5 culture to maintain cell survival.

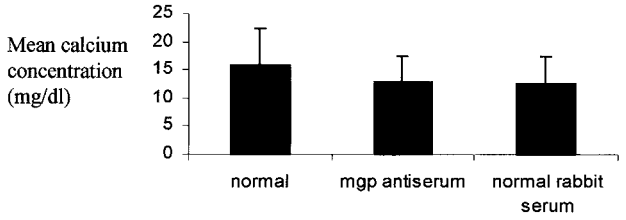

Figure 6. **Calcium levels in MGP antiserum–treated ATDC5 cells.** Calcium concentrations in ATDC5 cell cultures supplemented with MGP antiserum at 1:200 dilution, with normal rabbit serum at 1:200 dilution, and with no supplements.

Inducible overexpression of MGP in ATDC5 cells

To determine the effects of increased levels of MGP at various stages of the endochondral ossification pathway, we constructed a pIND vector containing a 428-bp fragment of mouse MGP cDNA subcloned into the EcoR1 and Xba1 sites of the plasmid in-frame in the sense (5′ to 3′) orientation. After dual transfection of ATDC5 cells with the pIND sense construct (pINDE/X) and pVgRXR, stably transfected clones were generated in the same way as for the antisense clones. Two clones, E/X3 and E/X5, were chosen for further analysis to confirm stable transfection. Inducible-specific expression of pINDE/X in the cells was determined by RT-PCR analysis (Fig. 2).

Clone E/X5, which expressed the inducible sense MGP cDNA, was plated out in multiple 6-well plates. Cultures were induced with ponasterone A from day 3 and at each medium change. Induction with ponasterone A increased the level of MGP mRNA in the cells, measured by real time PCR, to ~280% of the level in the noninduced cells. An increased level of expression was confirmed by Western blot analysis (Fig. 2). The progress of cultures was examined after induction at day 3 and at day 12. In both instances, the induced cultures grew normally, condensing, proliferating, and forming cartilage nodules until day 21, when they apoptosed and detached (Fig. 8). Noninduced, transfected cell control cultures continued to grow normally with normal matrix mineralization at day 42 (Fig. 8). These results suggest that overexpression of MGP has no deleterious effects on ATDC5 differentiation until day 21. This corresponds to the time when MGP expression falls, as shown by Northern analysis (Fig. 1). Real time PCR analysis confirmed raised MGP levels at multiple time points in the induced, transfected cells compared with their controls. In addition, there were no significant differences in type II collagen levels between the induced cells and controls. However, levels of type X collagen were significantly lower in the transfected cells ($P < 0.001$) compared with the controls. Therefore, the decrease in MGP levels during the maturing phase of ATDC5 differentiation is critical to allow chondrocyte passage through to hypertrophy.

ATDC5 cell cultures transfected with the pINDE/X vector were also induced at day 21 and thereafter with ponasterone A. These cultures grew normally through to day 42 with maintenance of cartilage nodules. However, alizarin red staining (Fig. 8) and measurement of calcium levels revealed marked diminution of matrix mineralization in the induced, transfected cultures compared with control cultures ($P =$

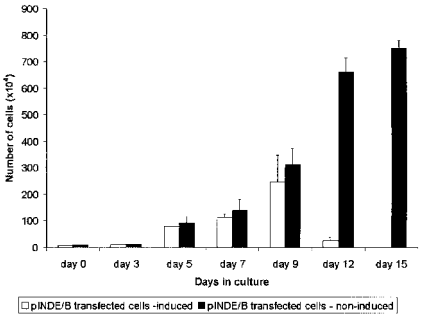

Figure 7. **Cell proliferation of ATDC5 cells transfected with MGP antisense construct.** Cell proliferation of ATDC5 cells transfected with pINDE/B. Cultures were induced with 5 μM ponasterone A from day 3 of culture or not induced. Cells were trypsinized and counted on a haemocytometer. At day 11, induced cells apoptosed and detached, whereas noninduced cells continued to proliferate and differentiate normally with normal matrix mineralization.

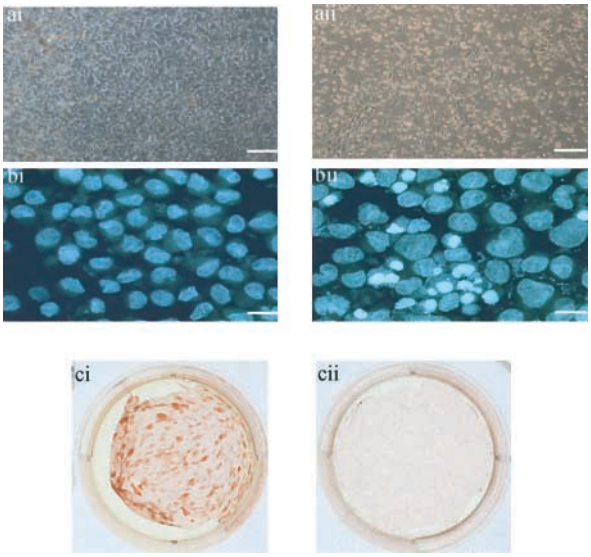

Figure 8. **Effects of overexpression of MGP in ATDC5 cells.** ATDC5 cells transfected with pINDE/X after 21 d of culture phase-contrast microscopy of (ai) noninduced cells and (aii) cells induced with 5 μM ponasterone A from day 3. Cells stained with Hoescht 33258 to determine nuclear morphology of (bi) noninduced cells and (bii) induced cultures showing nuclear condensation and fragmentation consistent with apoptosis. Alizarin red staining on day 42 of ATDC5 cells transfected with pINDE/X showing (ci) normal mineralization pattern and (cii) after induction from day 21 with 5 μM ponasterone A with reduced mineralization. Bars: (ai and aii) 120 μm; (bi and bii) 10 μm.

0.0008; Fig. 9). These findings support previous work which suggest that MGP functions as a cell-specific inhibitor of matrix mineralization (Luo et al., 1997; Yagami et al., 1999).

Discussion

MGP has a biphasic expression pattern in the ATDC5 chondrocyte system

We used the ATDC5 chondrocyte cell line to examine the significance of the biphasic expression of MGP within the mammalian growth plate and to explore whether MGP has functions beyond that of an inhibitor of matrix mineralization in hypertrophic chondrocytes (Luo et al., 1997; Yagami et al., 1999). The ATDC5 system is ideally suited to such experimentation, as the chondrocytes undergo a defined pattern of differentiation that resembles that within the mammalian growth plate (Shukunami et al., 1996; Enomoto et al., 2000). Further, we have shown by Northern analysis that expression is evident in the ATDC5 system MGP at day 9 of culture and increases at day 15 (corresponding to chondrocyte proliferation), then decreases at day 21 (corresponding to chondrocyte maturation/onset of hypertrophy), and increases again at day 32 (corresponding to the onset of matrix mineralization). Thus, the ATDC5 chondrocytes express MGP in a biphasic manner, similar to that in the growth plate in vivo (Luo et al., 1995), and thereby provide a means for examining whether MGP influences critical stages of the differentiation pathway.

Reduction in levels of MGP has an effect on proliferative and hypertrophic chondrocyte survival

Treatment of cultures with warfarin has previously been used to examine the role of MGP in chick proliferative and hypertrophic chondrocyte cell cultures (Yagami et al., 1999). However, the effects of warfarin are not specific for MGP, as it can directly alter the expression of a number of genes normally expressed in cartilage, as well as inhibit γ-carboxylation of Gla residues in other Gla-containing proteins apart from MGP (Barone et al., 1994). To circumvent these problems, we examined the effects of a reduction in MGP at distinct stages of differentiation after either treatment of cultures with an anti-MGP peptide antibody or induction of the expression of an MGP antisense transcript. We found that treatment of ATDC5 cultures with the MGP peptide antibody from day 3 of culture did not affect chondrocyte condensation, but induced their apoptosis by day 10 of culture. Inhibition of MGP expression in the ATDC5 cells by expression of the antisense MGP transcripts over this time period had an identical effect. These findings suggest that MGP is not required for chondrocyte condensation despite its abundant expression at a very early stage in mouse embryonic development in precartilagenous mesenchymal condensations (Luo et al., 1995). This is in keeping with the findings that in the MGP-deficient mouse, mesenchymal condensation and subsequent chondrocyte formation are not altered (Luo et al., 1997). The induction of chondrocyte apoptosis by inhibition of MGP in the ATDC5 cells coincided with the proliferation and cartilage nodule formation of untreated cultures. A role for MGP in the survival of chondrocytes at this stage of differentiation suggests that the disorganization of the proliferative chondrocyte zone in the MGP-deficient mouse is not due to aberrant calcification, but to aberrant chondrocyte proliferation (Luo et al., 1997). However, our findings contrast with those demonstrating that warfarin treatment of chick-proliferating chondrocytes had no adverse effect on their survival or proliferation (Yagami et al., 1999). This suggests that either MGP is not vital in the chick for proliferative chondrocyte survival, or that chondrocyte survival is not dependent on the Gla residue, which would be decarboxylated by the warfarin treatment. The fact that the MGP antiserum used in our studies was raised to a COOH-terminal peptide sequence of MGP

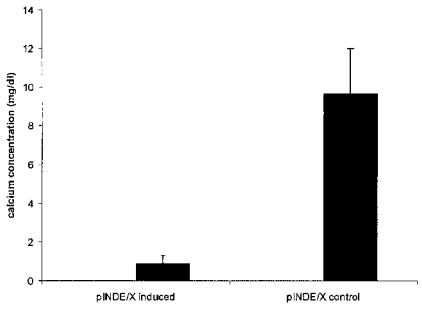

Figure 9. **Calcium levels in ATDC5 cells overexpressing MGP.** Calcium measurement in ATDC5 cell cultures transfected with pINDE/X on day 42. Cultures were induced with 5 μM ponasterone A from day 21 onwards or not induced as controls.

that does not contain any Gla residues supports this latter hypothesis. Further, the MGP antiserum had no effect on the mineralization of late stage ATDC5 cells, again suggesting that the antibody does not alter Gla residue function.

The demonstration that MGP antiserum has the same effect on chondrocyte survival as expression of the antisense MGP construct indicates that MGP affects this function at an extracellular location. The chondrocyte survival properties of MGP may be mediated via an as yet unidentified cell surface receptor analogous to the Gas6–Axl tyrosine kinase receptor interaction. Gas6, another member of the Gla-containing protein family, is able to protect serum-starved NIH3T3 cells and vascular smooth muscle cells (VSMC) from undergoing apoptosis. (Goruppi et al., 1996; Nakano et al., 1996). As inhibition of Gas6 activity with anti-Gas6 antibody or warfarin failed to induce VSMC death, this suggests that the cell survival properties of Gas6 are not Gla residue–dependent, similar to the effects noted with MGP (Nakano et al., 1996).

Once the ATDC5 chondrocytes had passed through the early proliferative phase, inhibition of MGP from either day 12 or 21 had no effect on chondrocyte nodule formation, but at day 26 (after chondrocyte hypertrophy but before mineralization) in both instances, the chondrocytes apoptosed and detached. This suggests that MGP is vital for hypertrophic chondrocyte survival. The effects of absent MGP on hypertrophic chondrocytes is not seen in the MGP-deficient mouse as hypertrophic chondrocytes are absent, presumably as they are unable to mature beyond the proliferative phase (Luo et al., 1997). In previous studies, warfarin has been shown to stimulate matrix mineralization of chick hypertrophic chondrocytes (Yagami et al., 1999). These results again suggest that the inhibition of mineralization by MGP is Gla residue–dependent, but that the cell survival properties conferred by MGP are not. Our results with the MGP antiserum in the hypertrophic ATDC5 cells are at variance with the cultures where MGP was inhibited by antisense MGP mRNA, which might reflect differences either in the accessibility, efficacy, or specificity of the antibody at this stage of culture as compared with the antisense MGP.

Inducible overexpression of MGP prevents chondrocyte maturation and inhibits mineralization

Previously, virally driven overexpression of MGP in chick limb buds has been demonstrated to cause severe alterations of limb skeletogenesis with disruption of chondrocyte maturation to hypertrophy (Yagami et al., 1999). In our studies, overexpression of MGP had no appreciable effect on early ATDC5 differentiation, allowing cellular condensation, chondrocyte proliferation, and cartilage nodule formation. However, corresponding to the stage when MGP levels decreased in noninduced cultures (at day 21), overexpression of MGP caused the cells to apoptose and detach. This suggests that the decrease in MGP levels that occurs in the maturing zone of the growth plate is vital to allow chondrocyte maturation and subsequent hypertrophy, and a failure in this modulation of expression disrupts this process. The lower levels of type X collagen in cells overexpressing MGP supports the suggestion that overexpression of MGP at this stage inhibits further chondrocyte differentiation. Overexpression of MGP has been found in association with apoptosis in rat glial cells stimulated with 1, 25-dihydroxyvitamin D3 (Baudet et al., 1998) and in the rat ventral prostate after androgen withdrawal (Briehl and Miesfeld, 1991). A fall in the transcription of MGP in serum-starved NIH3T3 cells suggested that the increase in MGP expression associated with apoptosis was not due to growth arrest (Briehl and Miesfeld, 1991). Our results suggest that this increase in MGP expression in association with apoptosis is more than a bystander effect and that MGP is able to stimulate apoptotic pathways either directly or in combination with other factors. When the bone morphogenetic proteins (BMPs) were first isolated, they were found to be in tight association with MGP (Urist et al., 1984). MGP has been characterized as a binding protein for BMP-2 (Wallin et al., 2000) and a recent report suggests that MGP modulates BMP-2 activity (Boström et al., 2001). BMP-2 has been characterized as an activator of apoptosis via the TAK1-p38 kinase pathway (Kimura et al., 2000), and it may well be that alterations of MGP levels at critical points in the endochondral pathway alter the interaction with BMP-2 and therefore affect apoptosis. This proposition will require further investigation. Overexpression in later stage differentiating ATDC5 cells caused a reduction in matrix mineralization consistent with previous experiments where virally induced overexpression of MGP in chick hypertrophic chondrocytes had a similar effect (Yagami et al., 1999). This confirms that MGP has a cell-specific function as an inhibitor of matrix mineralization.

Our results indicate that coordinated expression of MGP is critical for chondrocyte survival in ATDC5 cells, as inhibiting MGP expression during stages when it is normally expressed and overexpressing it when it is normally not expressed cause chondrocyte apoptosis. These findings imply that the biphasic expression pattern of MGP within the mammalian growth plate is required for normal chondrocyte differentiation within the growth plate. That excessive MGP should have the same effect as insufficient MGP at different stages in chondrocyte differentiation is intriguing. The mechanisms whereby MGP modules these effects remain to be elucidated.

Materials and methods

Cell culture

ATDC5 cells were a gift from Dr. A. Grigoriadis (King's College, London, UK) and were maintained as described previously (Atsumi et al., 1990).

Cells were cultured in a standard medium of DME/Hams' F12 (1:1) containing 5% FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (all GIBCO BRL), and 10 μg/ml human transferrin, 10 μg/ml bovine insulin, and 3×10^{-8} M sodium selenite (all Sigma-Aldrich) at standard conditions (37°C in a humidified atmosphere of 5% CO_2 in air). Cells were plated in 6-well plates at a density of 6×10^4 cells/well (day 0). Cells were grown continuously in standard medium, which was replaced on alternate days. After 21 d of culture, the medium was converted to αMEM (GIBCO BRL) with the same supplements and the CO_2 level was lowered to 3%.

Integrity of cultures was confirmed by phase contrast microscopy. Chondrogenesis was confirmed by fixing cells in 95% methanol and staining with in 0.5% alcian blue, pH 0.75 (Fluka), at various time points. To determine cell proliferation, cells were trypsinized and counted on a haemocytometer at fixed time points until day 15, when cells could not be completely disassociated by trypsinization.

Northern blot analysis

RNA was extracted from the ATDC5 cells at different time points by a previously described method (Chomczynski and Sacchi, 1987). Samples of total RNA (20 μg) were separated by electrophoresis on a 1% (wt/vol) agarose formaldehyde gel and transferred to nitrocellulose filters by vacuum blotting and baked in a vacuum oven at 80°C for 2 h. The filter was hybridized with radio-labeled cDNA inserts at 68°C in solution containing 1% (wt/vol) BSA, 7% (wt/vol) SDS, and 0.125× high salt buffer (1× high salt buffer contains 2 M NaCl, 1 M HNa_2O_4P, and 0.14 M H_3PO_4) washed, exposed, and developed using standard conditions (Sambrook et al., 1989). Hybridization cDNA probes were made by the random-primer method using the Oligolabeling kit (Amersham Pharmacia Biotech) using a 0.4-kb EcoR1-Xba1 fragment of pMGPcJ (clone was a gift from Professor G. Karsenty, Baylor College of Medicine, Houston, TX) (Luo et al., 1995) for mouse MGP; a 0.3-kb Xho1 fragment of IMAGE clone 1969710 (HGMP Resource Centre, Cambridge, UK) for mouse type X collagen; a 1.3-kb Not1-Sal1 fragment of IMAGE clone 613911 for mouse type II collagen; a 0.45-kb Not1-EcoR1 fragment of IMAGE clone 472468 for mouse osteocalcin; and a 1.5-kb Not1-Sal1 fragment of IMAGE clone 3156074 for mouse cytoplasmic β-actin.

Antibody inhibition studies

ATDC5 cells were plated at 6×10^4 cells/9.4 cm^2 well in 6-well plates and grown in standard medium. To each well of six 6-well plates on day 3, filter-sterilized MGP antiserum at 1:200 dilution was added and at each medium change thereafter. The MGP antiserum used was rabbit antiserum against a synthetic peptide of the COOH-terminal sequence (ERYAMVYGYNAAYNRYFRQRRGAKY) of mouse MGP (donated by Professor G. Karsenty, Baylor College of Medicine, Houston, TX). To three separate 6-well plates, MGP antiserum was added from day 21 and at each medium change thereafter. As controls, ATDC5 cell cultures were grown concurrently with no antibody supplements and with filter-sterilized normal rabbit serum (Sigma-Aldrich) at a 1:200 dilution added at the same time points as the MGP antiserum. These experiments were performed on at least three separate occasions.

Construction of sense and antisense MGP-inducible expression vectors

The ecdysone-inducible mammalian expression system (Invitrogen) relies on the constitutive expression of a heterodimeric ecdysone receptor from *Drosophila* by the regulator vector pVgRXR. The ecdysone-responsive promoter is located in a second vector, pIND, containing the gene of interest. In the presence of the inducer, ponasterone A (Invitrogen), the functional ecdysone receptor binds to the promoter and activates gene expression.

A 398-bp cDNA fragment of mouse MGP was excised from pMGPcJ using EcoR1 and Xba1 and directionally cloned into the EcoR1 and Xba1 sites in the multiple cloning site of pIND. The MGP cDNA in this construct (pINDE/X) is in the sense orientation with regard to the upstream promoter. For the antisense construct (pINDE/B), the MGP cDNA was excised from pMGPcJ using EcoR1 and BamH1 and directionally cloned into the EcoR1 and BamH1 sites of the multiple cloning site of the pIND vector. The orientation and sequence fidelity of the clones were checked by restriction enzyme digest reactions, generating 416- and 428-bp fragments for MGP cDNA within pINDE/B and pINDE/X, respectively, and DNA sequencing using Big Dye terminator cycle sequencing reactions and analysis on an ABI PRISM 377 DNA Sequencer (PerkinElmer).

Transfection and selection of stable cell lines

Dual transfections with pVgRXR and the respective pIND constructs were performed using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. After transfection, cells were maintained in standard medium minus the ITS, but containing Zeocin™ (400 μg/ml of medium; Invitrogen) and G418 (300 μg/ml of medium; Sigma-Aldrich). Antibiotic-resistant clones transfected with either pINDE/X or pINDE/B were selected after 14 d by ring cloning (McFarland, 2000). The clones were expanded in standard medium, including ITS, Zeocin, and G418. To confirm that the plasmid constructs affected over- and underexpression of MGP in the clones an aliquot of cells from each clone was induced with 5 μM ponasterone for 48 h after confluence. Both total RNA and protein were extracted from induced and noninduced control cultures, and RT-PCR analysis and Western blotting were performed as described. Stably transfected clones were plated at 6×10^4 cells/well in 6-well plates and grown in standard medium with Zeocin and G418 supplementation. Nine concurrent cultures were induced with 5 μM ponasterone at days 3, 12, and 21, with the addition of the inducing agent at each medium change thereafter. Noninduced, transfected cells were grown in parallel as controls. In addition, 5 μM of ponasterone was added to normal ATDC5 cells from day 3 to 42 as a control. All cell culture experiments were performed on a minimum of three occasions.

Reverse transcriptase (RT)-PCR

Mouse MGP transcripts were amplified by RT-PCR to confirm over- and underexpression of MGP in the transfected cell lines. Total RNA was treated with 5 μg DNase 1 and incubated at 37°C for 1 h. The RNA (1 μg) was reverse transcribed using 0.5 μg oligo $(dT)_{15}$, 5 mM dNTPs (Sigma-Aldrich), 40 U RNAse inhibitor, 25 U AMV reverse transcriptase, and 1.25× reverse transcriptase buffer (all Roche Molecular Biochemicals) at 42°C for 1 h and at 90°C for 3 min. Amplification of 1/10 of the cDNA reaction was carried out with 15 pmol primers, 0.1 mM each of dATP, dCTP, dGTP, dTTP, 5 U Taq polymerase, 1× Taq polymerase buffer (Roche Molecular Biochemicals). The following cycling conditions were used: to denature, 96°C for 60 s; to anneal, 59°C for MGP primers and 58°C for β-actin primers for 90 s; and 72°C for 60 s for 23 or 35 cycles on a DNA Thermal Cycler (Cetus 480; PerkinElmer). The sequences of primers for mouse MGP cDNA were (mgpF) 5′-ATGAAGAGCCTGCTCCCTCT-3′ and (mgpR) 5′-ATATTTGGCTCCTCGGCGCT-3′ generating a 312-bp fragment (Ikeda et al., 1991). To confirm inducible expression from the pINDE/X construct, the ecdysone forward primer sequence 5′-CTCTGAATACTTTCAACAAGTTAC-3′ and primer mgpR were used to generate a 450-bp fragment. For the pINDE/B construct with the ecdysone, forward and mgpF primers were used to generate a 471-bp fragment. The sequences of mouse cytoplasmic β-actin primers were 5′-GAAGGCGACAGCAGTTGGTT-3′ and 5′-ACGCGACCATCCTCCTCTTA-3′, generating a 204-bp fragment (Tokunaga et al., 1986).

Real time PCR

Mouse MGP transcripts were amplified by real time PCR to establish the levels of over- and underexpression of MGP in the transfected cell lines. Total RNA was extracted using TRIZOL (GIBCO BRL) and treated with DNase 1 at 37°C for 30 min. The RNA (2 μg) was reverse transcribed using the Taqman Gold RT-PCR kit according to the manufacturer's instructions: 2.5 μM oligo $(dT)_{16}$, 500 μM (of each) dNTPs, 0.5 U/μl RNase inhibitor, 1.25 U/μl MultiScribe reverse transcriptase, 1× TaqMan reverse transcriptase buffer at 25°C for 10 min, 48°C for 20 min and 95°C for 5 min (PE Biosystems). Amplification of 1/30 of the cDNA reaction was carried out using the SYBR green PCR method according to manufacturer's instructions (PE Biosystems). In brief, 1× SYBR green master mix, 300 nM forward primer and 300 nM reverse primer and the cDNA were heated at 95°C for 10 min. The following cycling conditions were used: 95°C for 15 s 60°C for 1 min for 40 cycles on the PE-ABI 7700 sequence detector. cDNAs were amplified using the following primers: ColII F, 5′-GGTGGCTTCCACTTCAGCTAT-3′; ColII R, 5′-TCATTGGAGCCCTGGATGAG-3′; ColX F, 5′-TTCTCCTACCACGTGCATGTG-3′; ColX R, 5′-AGGCCGTTTGATTCTGCATT-3′; MgpF, 5′-CCTGTGCTACGAATCTCACGAA-3′; MgpR: 5′-TCGCAGGCCTCTCTGTTGAT-3′; β-actin F, 5′-GAAGGCGACAGCAGTTGGTT-3′; β-actin R, 5′-ACGCGACCATCCTCCTCTTA-3′; Bcl-2 F, 5′-TGTGTGTGGAGAGCGTCAACA-3′; Bcl-2 R, 5′-CCAGGTATGCACCCAGAGTGAT-3′; Bax F, 5′-AGCTGCAGAGGATGATTGCT-3′; Bax R, 5′-GATCAGCTCGGGCACTTTAG-3′. All quantitations were normalized to β-actin as an endogenous control and relative to a calibrator. All real time PCR experiments were performed four times. Statistical analysis was performed using a two-tailed Student's *t* test.

Western blotting

Protein was extracted from ATDC5 cell cultures by the direct addition of 1× SDS sample buffer (0.5% [wt/vol] SDS, 2.5% [vol/vol] glycerol, 15.6 mM Tris-HCl, pH 6.8, 0.125% [vol/vol] bromophenol blue, 0.5% [vol/vol] β-mercaptoethanol). Protein was separated by 15% (wt/vol) SDS-PAGE

(Laemmli, 1970) alongside standard color markers. The gel was electroblotted to nitrocellulose in Towbin transfer buffer (Towbin et al., 1979). Equal and efficient transfer of protein was confirmed by staining the nitrocellulose with Ponceau S (0.2% [wt/vol] Ponceau S/3% [vol/vol] trifluoroacetic acid). The MGP antiserum at 1:200 was used as primary antibody and incubated overnight at 4°C. A biotinylated swine anti–rabbit secondary antibody (Dako) was used at 1:3,000 dilution and incubated for 30 min at room temperature. The chemiluminescence (ECL®) detection system was used to visualize the result (Amersham Pharmacia Biotech).

Measurement of precipitated in vitro calcium

To determine the level of matrix mineralization, cultures were washed in PBS Dulbecco's (GIBCO BRL), fixed in 50–100% methanol, and stained with 1% (wt/vol) alizarin red, pH 4.2 (Fluka).

Separate cell cultures were decalcified by overnight incubation in 0.6 N HCl. The calcium content of the HCl supernatant was determined colorimetrically by the o-cresolphthalein complexone method (Sigma-Aldrich; Mori et al., 1998). Statistical analysis of calcium levels was performed using a two-tailed Student's *t* test.

Apoptosis

Markers of apoptosis were determined in two ways. Bcl-2 and Bax levels were established at various time points by real time PCR as described above.

Secondly, nuclear morphology of the ATDC5 cells was examined by fixing the chondrocytes in 1% (vol/vol) formaldehyde. The cells were stained with 0.5 μg/ml Hoechst 33258 and then visualized by fluorescence microscopy (Pullan et al., 1996).

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