Matrix Gla protein is differentially expressed during the deposition of a calcified matrix by vascular pericytes

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Abstract PCR-based subtractive hybridisation was used to identify genes up-regulated when pericytes undergo osteogenic differentiation and deposit a calcified matrix. cDNA pools were generated from confluent pericytes and from pericyte cultures containing calcified nodules. A pericyte cDNA library was screened with the product of the subtraction procedure (calcified minus confluent cDNA) and the majority of the positive clones were identified as matrix Gla protein (MGP). Northern analysis and immunohistochemistry demonstrated that MGP was only expressed by pericytes in calcified nodules. Antibodies to MGP inhibited the deposition of a calcified matrix by pericytes, suggesting that MGP regulates both cell differentiation and calcification.

Key words: Matrix Gla protein; Pericyte; Calcification

1. Introduction

Pericytes are an integral part of the microvasculature, where they partially surround endothelial cells sharing a common basement membrane [1]. Pericyte-like cells have also been localised in the sub-endothelial and medial layers of large, medium and small human arteries as well as in the vasa vasora of the adventitia [2]. There is good evidence to suggest that pericytes have osteogenic potential in vivo and in vitro, and it has been proposed that these cells may play a role in the growth, maintenance and repair of the skeleton and in diseases involving ectopic calcification [3–6]. Indeed, several recent studies have highlighted the potential role of pericytes, or pericyte-like cells present in arteries, in mediating the calcification associated with atherosclerosis [7,8].

Vascular pericytes display a distinctive and reproducible pattern of growth and differentiation in vitro [6]. After reaching confluence, these cells form multicellular nodules. Crystals of hydroxyapatite are then deposited on the extracellular matrix of these nodules [3,4]. We have demonstrated that this process is associated with the stage-specific expression of markers of primitive clonogenic marrow stromal cells (STRO-1), and markers of cells of the osteoblast lineage (bone sialoprotein, osteocalcin, osteonectin, osteopontin and type I collagen) [5,6]. However, the regulation of pericyte differentiation is by no means understood.

In this study we have used PCR-based subtractive hybridisation to identify genes that are switched on as pericytes differentiate into bone-forming cells and deposit a calcified matrix. The majority of the positive clones were identified as matrix Gla protein (MGP), a small Gla-containing protein previously shown to accumulate in adult bone, cartilage and dentin [9]. We demonstrate that MGP is differentially expressed during the differentiation of pericytes in vitro, and that antibodies to MGP inhibit the deposition of a calcified matrix by these cells. These results are discussed in terms of the proposed roles of MGP in regulating cell differentiation and calcification.

2. Materials and methods

2.1. Cells and culture conditions

Pericytes were isolated from adult bovine retinal microvessels, characterised and cultured as previously described [5]. The results presented herein were obtained using 20 different preparations of bovine retinal pericytes at first or second passage.

2.2. RNA isolation and analysis

RNA was isolated from (i) sub-confluent pericytes (day 4 post-plating), (ii) confluent pericytes (days 7–10 post-plating), (iii) post-confluent cultures containing small non-calcified nodules (days 14–25 post-plating), (iv) post-confluent cultures containing calcified nodules (days 30–45 post-plating) and (v) from purified pericyte nodules [5].

2.3. Amplification of cDNA

PolyA cDNA with an average length of 400 bases was generated using the purified pericyte RNA as described [5,10].

2.4. Subtractive hybridisation

Subtractive hybridisation was performed to isolate genes that were up-regulated during the osteogenic differentiation of vascular pericytes [10]. A 20-fold molar excess of polyA cDNAs prepared from confluent pericytes was photobiotinylated and mixed with non-biotinylated target cDNAs prepared from post-confluent pericytes. After boiling to denature the double-stranded DNA and hybridisation, all biotinylated hybrids were removed using avidin/organic extraction. Residual target cDNA was amplified and subjected to four further rounds of subtraction. The polyA cDNA obtained from the final round of the subtraction procedure was used to screen a custom-made Lambda ZAP-II cDNA library prepared from pericyte mRNA extracted from cultures containing cells at all stages of their growth and differentiation (Stratagene Ltd, Cambridge, UK). Filters were hybridised overnight at 37°C with a ^{32}P-labelled subtracted cDNA probe. The filters were washed with 0.5×SSC/0.1% SDS at 37°C, and exposed to X-ray

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film (Fuji) at −80°C with intensifying screens. Purified positive phagemids were isolated from the Lambda ZAP II vector using the ExAssist/SOLR system (Stratagene). Plasmids of interest were purified and sequenced on an ABI Prism 377 sequencer using a PCR-based dye terminator sequencing kit (Perkin Elmer).

2.5. Random labelling of cDNA probes

Hybridisation probes were prepared by random labelling 50 ng DNA with [α-^{32}P] dCTP using an oligolabelling kit (Amersham Pharmacia Biotech, UK).

2.6. Southern blotting

Amplified cDNAs (5–10 μl per lane) were electrophoresed in 1.5% agarose in Tris-borate buffer, denatured in NaOH/NaCl, neutralised in Tris–HCl/NaCl and transferred to Hybond N nylon membranes. Tracks containing control samples for the PCR reaction did not stain with ethidium bromide. Filters were hybridised with ^{32}P-labelled probes at 65°C overnight, washed at high stringency (0.2×SSC, 0.1% SDS at 65°C) and exposed to X-ray film at −80°C.

2.7. Northern blotting

Samples of RNA (15 μg) were separated in 1.2% agarose gels containing 2.2 M formaldehyde, transferred to Hybond N nylon membranes and fixed by baking for 1 h at 80°C. Filters were pre-hybridised and hybridised with ^{32}P-labelled probes at 65°C in phosphate buffer containing 1% BSA and 7% SDS [11]. Filters were washed in 2×SSC, 1% SDS at 65°C for 15 min, in several washes of 0.2×SSC, 0.1% SDS at 65°C for 1 h and exposed to X-ray film. This procedure was repeated using RNA isolated from three different preparations of pericytes.

2.8. Confirmation of specificity of anti-MGP serum

A rabbit polyclonal antibody raised against a C-terminal MGP peptide (ERYAMVYGYNAAYNRYFRQRRGAKY) was kindly provided by Dr G Karsenty (Houston, TX, USA). This antibody has previously been shown to be specific for MGP by ELISA (Dr G. Karsenty, personal communication). The specificity of this antibody was confirmed by Western blotting. Growth plate and resting zone (epiphyseal) chondrocytes were isolated from second trimester bovine foetuses, separated by SDS–PAGE using a 12.5% gel under reducing conditions, and transferred to nitrocellulose as described [12]. Efficient transfer of protein was confirmed by soaking the nitrocellulose filter in 0.2% (w/v) Ponceau S/3% (v/v) trifluoroacetic acid for 5 min. The filter was blocked for 2 h in 1×PBS/0.05% (v/v) Tween 20 (PBS-T)/5% (w/v) non-fat dried milk. Primary antibody was added at 1:250 dilution and incubated for 2 h at room temperature. The filter was washed with PBS-T and then incubated with a horseradish-peroxidase-conjugated swine anti-rabbit secondary antibody (1:2000 dilution, Dako, Denmark) for 1 h at room temperature. The filter was washed with PBS-T and the manufacturer's instructions followed for ECL™ detection (Amersham Pharmacia, St Albans, UK). The result was visualised by exposure of the membrane to X-ray film (Kodak) and developed using a Kodak automatic developer.

2.9. Immunolocalisation of MGP in pericyte nodules

Pericytes were cultured in standard culture medium until large, calcified nodules were present. Nodules were collected from the base of the flask using a sterile needle, snap-frozen and sectioned. Cryostat sections (10 nm) were air-dried for 24 h, fixed in acetone (10 min), air-dried for 40 min and then stored at −80°C until use.

Immunohistochemistry was performed using a standard streptavidin biotin immunoperoxidase method (Duet kit, Dako, UK). The anti-MGP antibody was used at a dilution of 1:200. Sections were counterstained with haematoxylin. Negative controls included substitution of the primary antibody with normal rabbit serum.

2.10. Addition of anti-MGP sera to pericyte cultures

Cultures were incubated in the presence of specific antibodies against MGP, as previously described [11]. Briefly, pericytes (2×10^4 cells/cm^2) were plated in 2 cm^2 dishes, and cultured in standard growth medium until (a) the cells were 80% confluent or (b) small, non-calcified nodules were present. Anti-MGP antiserum was then added to the culture medium (2 and 5 μl antiserum per ml medium), and the incubation continued for up to 33 days. Fresh antiserum was added every time the medium was changed. Normal rabbit serum (5 μl per ml medium) was added to control wells. Cells were stained with von Kossa, and both the total number of nodules present and the number of calcified nodules per culture was counted [11].

3. Results

3.1. Identification of genes up-regulated during pericyte differentiation

RNA was isolated from confluent pericytes and from post-confluent cultures of cells containing calcified nodules. PolyA PCR was performed to produce cDNA representative of the entire polyadenylated mRNA present in the two samples. Subtractive hybridisation was then conducted to isolate cDNAs which were specifically up-regulated in the post-confluent cultures. Amplified cDNA from confluent pericytes (Fig. 1, track 1), post-confluent pericytes (Fig. 1, track 2) and the four subtraction products (S1–S4; Fig. 1, tracks 3–6) was electrophoresed on an agarose gel and subjected to Southern hybridisation using ^{32}P-labelled cDNA probes. The ethidium bromide-stained gel (Fig. 1A) shows the amount of cDNA loaded into each track of the agarose gel. Fig. 1B shows the results obtained when this gel was probed with S4 cDNA. This probe hybridised with samples in tracks 2–6, but not with the sample in track 1 (from confluent pericytes). In comparison, the sample in track 1 hybridised strongly with a confluent pericyte cDNA probe (Fig. 1C). In addition, the confluent pericyte cDNA probe hybridised with the sample in track 2 (from post-confluent pericytes), demonstrating that these two samples have many cDNAs in common. In contrast, the confluent cDNA probe hybridised only very weakly with the four subtraction products (tracks 2–6), confirming that the majority of shared sequences were removed by this procedure.

The subtracted cDNAs were then used to screen a pericyte

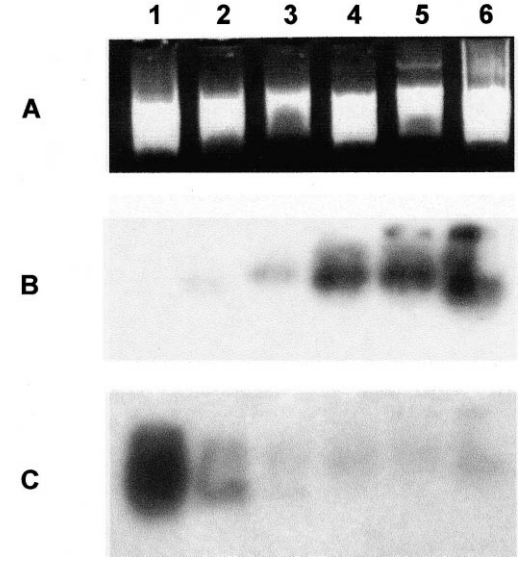

Fig. 1. Specificity of the subtraction product for poly(A) cDNAs prepared from post-confluent cultures of pericytes containing calcified nodules. Samples of confluent poly(A) cDNA (track 1), post-confluent poly(A) cDNA (track 2) and subtracted poly(A) cDNAs (S1–S4, tracks 3–6 respectively) were electrophoresed on a 1.5% agarose gel, visualised with ethidium bromide (A), and subjected to Southern hybridisation with ^{32}P-labelled S4 cDNA (B) or confluent cDNA (C).

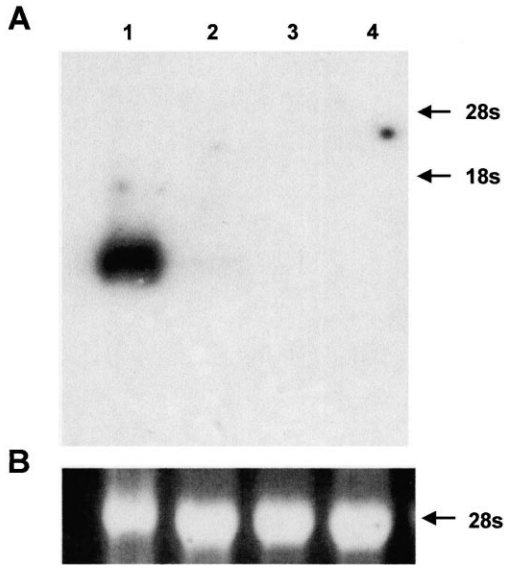

Fig. 2. Expression of MGP during pericyte differentiation. RNA from post-confluent pericytes containing large calcified nodules (track 1), post-confluent pericytes containing small non-calcified nodules (track 2), confluent pericytes (track 3) and sub-confluent pericytes (track 4) was subjected to Northern analysis. A: Hybridisation with ^{32}P-labelled cDNA probe for MGP. B: Ethidium bromide-stained gel. The positions of 28S and 18S ribosomal RNA are indicated.

cDNA library. Positive plaques were identified, collected and purified for further analysis as described in Section 2. One clone, which hybridised strongly with the S4 cDNA probe, was found to contain an insert of approximately 600 bp. This clone was identified as MGP by PCR-based dye terminator cycle sequencing.

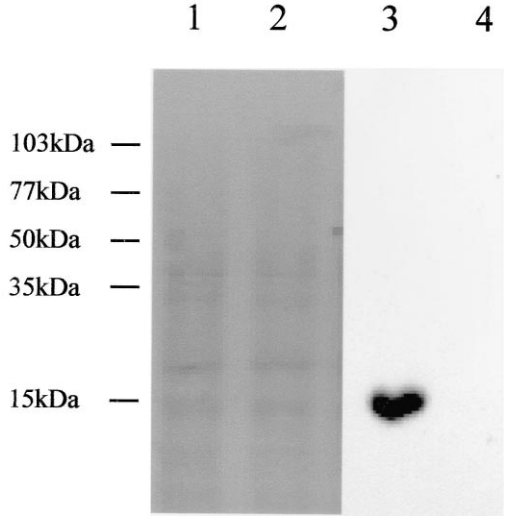

Fig. 3. Western blot demonstrating the specificity of anti-MGP sera. Lysates of bovine proliferative zone chondrocytes (tracks 1 and 3) and resting zone chondrocytes (tracks 2 and 4) were separated by SDS–PAGE and subjected to western blotting using anti-MGP sera (tracks 3 and 4). Tracks 1 and 2 demonstrate equal transfer of protein onto the nitrocellulose filter by Ponceau S staining. Track 3 demonstrates a single band at approximately 15 kDa (the predicted weight of bovine MGP). There is no corresponding band in track 4, indicating absence of MGP in resting zone chondrocytes. The prestained molecular weight markers are indicated on the left.

3.2. *Confirmation that MGP is differentially expressed by cultured pericytes*

The level of MGP mRNA in cultured pericytes was examined by Northern blotting. RNA prepared from pericyte cultures at different stages of their growth and differentiation was electrophoresed on an agarose gel and subjected to Northern hybridisation using a cDNA probe for MGP. Fig. 2A confirms that MGP is only expressed by cells present in post-confluent cultures containing large calcified nodules. MGP mRNA was not detected in sub-confluent pericytes, in confluent pericytes or in pericyte cultures containing small non-calcified nodules. Fig. 2B confirms that similar levels of RNA were loaded on each track of the agarose gel.

To confirm that MGP was expressed by cells presents in pericyte nodules, immunolocalisation studies using specific antisera to MGP were performed. The specificity of this antibody was confirmed using Western blotting and immunohistochemistry. Fig. 3 clearly demonstrates that the MGP antiserum reacts with a 15 kDa protein (approximately) in proliferative zone chondrocytes, but not in resting zone chondrocytes (compare tracks 3 and 4). Furthermore, this antibody specifically stains chondrocytes present in the proliferating and late hypertrophic zone of bovine growth plate cartilage, and does not stain cells present in the zone of maturation (results not shown). These data are consistent with previous studies describing the localisation of MGP in cartilage [13,14]. Pericyte nodules were collected, snap-frozen, sectioned and stained using this antiserum. Staining for MGP was seen throughout the calcified pericyte nodules (Fig. 4B). Controls in which the first antiserum was substituted with normal rabbit serum were negative (Fig. 4A).

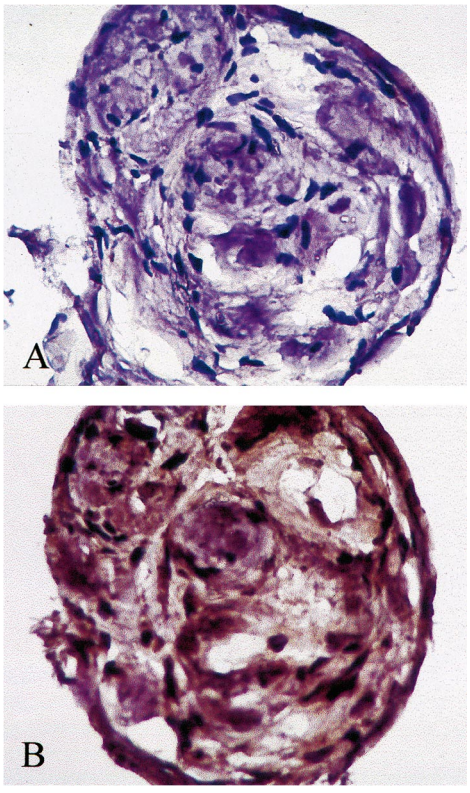

Fig. 4. Immunolocalisation of MGP in pericyte nodules. A: Negative control. B: Section stained with antibodies to MGP.

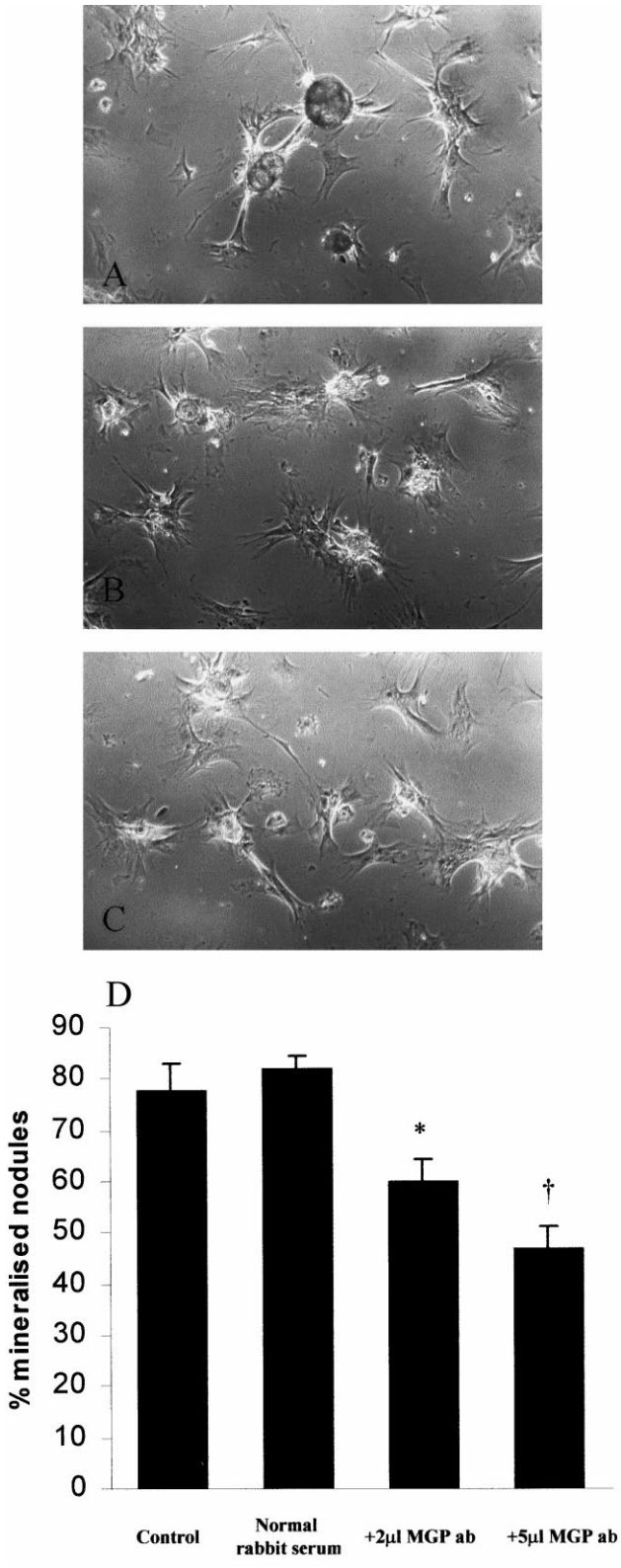

Fig. 5. Effect of antibodies to MGP on the production of a calcified matrix by pericytes. A: Control cells cultured in the presence of normal rabbit serum (5 μl). B: Cells cultured in the presence of 2 μl anti-MGP serum or (C) 5 μl anti-MGP serum. D: The total number of nodules present in the cultures and the percentage of these nodules that were calcified were determined. *$P < 0.05$ vs control and normal rabbit serum. †$P < 0.001$ vs control and normal rabbit serum.

3.3. Antibodies to MGP inhibit calcification of pericyte nodules

In the next series of experiments, the role of MGP in the deposition of a calcified matrix by pericytes was investigated. Antibodies to MGP were added to either confluent pericytes or cultures of pericytes containing non-calcified nodules, and the effects of these antibodies on the number of nodules formed and the extent of calcification of these nodules determined. Normal rabbit serum was added to parallel cultures as a control.

Representative photomicrographs of cells incubated in the presence and absence of antisera to MGP and stained with von Kossa (which stains mineral deposits brown/black) are shown in Fig. 5. These micrographs demonstrate clear differences in the extent of calcification of pericyte nodules in control cultures (Fig. 5A) compared with those cultures incubated with antisera to MGP (Fig. 5B,C). This experiment was repeated three times with two different populations of pericytes. The total number of nodules formed and the extent of calcification of these nodules were quantified as described in Section 2, and the results obtained from a representative experiment are shown in Fig. 5D. Antisera to MGP did not affect the total number of nodules formed in these cultures. However, antiserum to MGP reproducibly, and significantly, reduced the extent of calcification of nodules in pericyte cultures.

4. Discussion

Several lines of evidence suggest that MGP is a key regulator of both cell differentiation and calcification [14,15]. Firstly, MGP is required for endochondral ossification as it has been demonstrated that maturation of the growth plate is disrupted if MGP is either deleted or over-expressed in vivo [14,15]. Secondly, MGP inhibits the calcification of cartilage. Thus, the targeted deletion of MGP in mice results in the spontaneous calcification of the entire cartilage growth plate [14] and over-expression of MGP in chick limb buds inhibits cartilage calcification [15]. Furthermore, patients with Keutel syndrome, which is caused by mutations in the gene encoding MGP, have abnormal cartilage calcification [16]. Thirdly, it has been claimed that the effect of MGP on matrix calcification is dependent upon the stage of differentiation of the cells, suggesting that this protein may have other roles in immature cells [15].

The role of MGP in regulating calcification of arteries is less well understood. Firstly, mice deficient in MGP develop normally but die shortly after birth as a result of medial calcification of all major arteries leading to blood vessel rupture [14]. However, patients with Keutel syndrome have a normal life expectancy with no increased risk of coronary artery disease in the absence of functional MGP, suggesting that medial calcification is regulated by another mechanism in humans [16]. Secondly, the level of expression of MGP in normal and calcified human arteries appears to be dependent upon the type of calcification present. Thus, MGP is detected at low levels in normal blood vessels and is increased in patients with atherosclerosis where it is associated with calcium and lipid in the intima [17]. In contrast, patients with medial calcification express lower levels of MGP than normal, although the expression of this protein is focally increased in a subset of smooth muscle cells adjacent to sites of calcification [18]. Finally, the levels of MGP have been reported to

both decrease [19] and increase [20,21] during the calcification of vascular smooth muscle cells and calcifying vascular cells in vitro. The reason for this discrepancy is not clear.

In this study, we have demonstrated that MGP is differentially expressed when pericytes deposit a calcified matrix in vitro. Using Northern blotting and immunohistochemical staining of pericyte nodules, we have confirmed that MGP is only expressed in pericyte cultures containing large, calcified nodules. MGP is not expressed by confluent pericytes. These data are consistent with the studies of Tintut et al. [20] and Proudfoot et al. [21] who demonstrated that the levels of MGP increased as cells isolated from large blood vessels deposited a calcified matrix, and these groups suggested that (i) MGP was up-regulated in order to inhibit the extent of calcification or (ii) MGP inhibited calcification until the nodules developed to a form that facilitated calcium deposition [20,21].

The results of our functional studies using antibodies to MGP now suggest an alternative explanation. If the sole function of MGP is to inhibit matrix calcification, as suggested by the gene deletion studies in mice, the addition of anti-MGP serum to cultured cells should promote matrix calcification as these antibodies have been shown to have the same function-blocking effect as anti-sense MGP in cultured chondrocytes (Newman, W., Wallis, G.A. and Grant, M.E., unpublished information). However, our studies demonstrate that antibodies to MGP inhibit the deposition of a calcified matrix by pericytes. These results support the suggestion that MGP has a dual role in vascular cells, firstly modulating cell differentiation and subsequently controlling matrix calcification. If this is the case, antibodies to MGP may prevent pericytes from reaching the point at which they calcify their matrix. One possible explanation for this effect is that the antibodies inhibit the interaction between MGP and bone morphogenetic proteins [22], which are synthesised by pericytes (Canfield, A.E., unpublished observations) and calcifying vascular cells [7]. Therefore, the evidence presented here and elsewhere [15,16] suggest that the function of MGP is not as straightforward as originally proposed and further studies are needed to fully define the mechanism by which MGP modulates both vascular cell differentiation and matrix calcification.

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