

Expression of the gene encoding the matrix gla protein by mature osteoblasts in human fracture non-unions

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Abstract

Background—Osteoblast phenotypic abnormality, namely the expression of collagen type III, has been shown previously in fracture non-union woven bone.

Aims—To investigate osteoblasts from fracture non-unions for evidence of gene expression of non-collagenous bone matrix proteins that have been implicated in mineralisation, namely matrix gla protein (MGP), osteonectin, osteopontin, and osteocalcin. MGP is a consistent component of bone matrix, but there are no reports of osteoblasts in the skeleton expressing the gene for MGP, and the site of synthesis of skeletal MGP (perhaps the liver) has yet to be determined.

Methods—Biopsies from normally healing human fractures and non-unions were examined by means of in situ hybridisation, using ³⁵S labelled probes and autoradiography to disclose levels of gene expression.

Results—In normally healing fractures, mature osteoblasts on woven bone were negative for MGP mRNA, but positive for osteonectin, osteopontin, and osteocalcin mRNA molecules. In non-unions, osteoblasts displayed a novel phenotype: they were positive for MGP mRNA, in addition to osteonectin, osteopontin, and osteocalcin mRNA molecules.

Conclusions—Mature osteoblasts in slowly healing fractures have an unusual phenotype: they express the gene encoding MGP, which indicates that control of osteoblast gene expression in non-unions is likely to be abnormal. This might be of importance in the pathogenesis of non-uniting human fractures, and is of current interest given the emerging status of MGP as an inhibitor of mineralisation.

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Keywords: fracture non-union; osteoblast; woven bone; in situ hybridisation; matrix gla protein; osteonectin; osteopontin

Several non-collagenous bone matrix proteins, including matrix gla protein (MGP), osteonectin/SPARC, osteopontin (formerly SPP1), and osteocalcin (formerly BGP (bone gla protein)) are found in large quantities in bone. They are thought to play a variety of important roles in bone development, growth, and turnover and are also thought to be involved in fracture repair. Osteocalcin appears to be unique to bone and dentine, and has been

used widely as a marker of bony tissue development. Osteonectin, osteopontin (both functional members of the thrombospondin family), and osteocalcin appear to have roles in mineralisation; in addition, osteopontin and osteocalcin seem to have functions in resorption.^{1,2} During skeletogenesis, expression of the gene encoding MGP (a protein originally described in bone matrix) has been used as a reliable marker of the chondrogenic lineage,³⁻⁵ with osteoblasts appearing uniformly negative.

In the process of fracture healing, the evidence published to date on non-collagenous bone matrix proteins is solely from an animal model using the rat femur.⁶⁻⁸ Although this system has been used widely as a model of human fracture healing, there are some differences: cartilage production in human fracture callus appears to be less exuberant, and the process of callus mineralisation appears to take place later in humans than in the rat. The importance of open epiphyses in the adult rat to chondrocyte behaviour in this model is unclear, but fracture repair in children (who have open epiphyses) exhibits clear differences from the process in adults. We studied the expression of the genes encoding MGP, osteonectin, osteopontin, and osteocalcin to determine whether woven bone development in normally healing human fractures is similar to that described in the rat, and whether differences existed in fracture non-unions compared with normally healing bone.

Materials and methods

TISSUE PREPARATION

Specimens of human fracture callus from normally healing fractures were taken when available from the fracture sites of 15 closed fractures during surgery carried out to treat malreduction that had developed during conservative treatment. Biopsies were obtained between one and four weeks after fracture. Patients were aged between 18 and 87 years and were otherwise fit. On subsequent follow up, to one year, all these fractures, classed as normally healing, were found to have united normally. Abnormally healing fracture callus from non-unions was taken from the fracture site of 12 patients with extra-articular ununited fractures between four and 48 months after fracture.

The biopsy specimens were fixed in 10% neutral buffered formalin, decalcified in 20% EDTA (pH 7.2) until decalcification was radiologically complete, embedded in paraffin wax, and sectioned at 7 µm.

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IN SITU HYBRIDISATION (ISH)

The probes used for ISH analysis of human osteonectin, osteopontin, and MGP were obtained from the American Type Culture Collection (ATCC), and that for osteocalcin

was obtained from Dr PJ Barr (Chiron Corporation, Emeryville, California, USA). Details of these primers are as follows: osteonectin, clone HHCH67, restriction digest insert ECORI 1.2 kb⁹; osteopontin, clone Op-30, 1.4 kb¹¹;

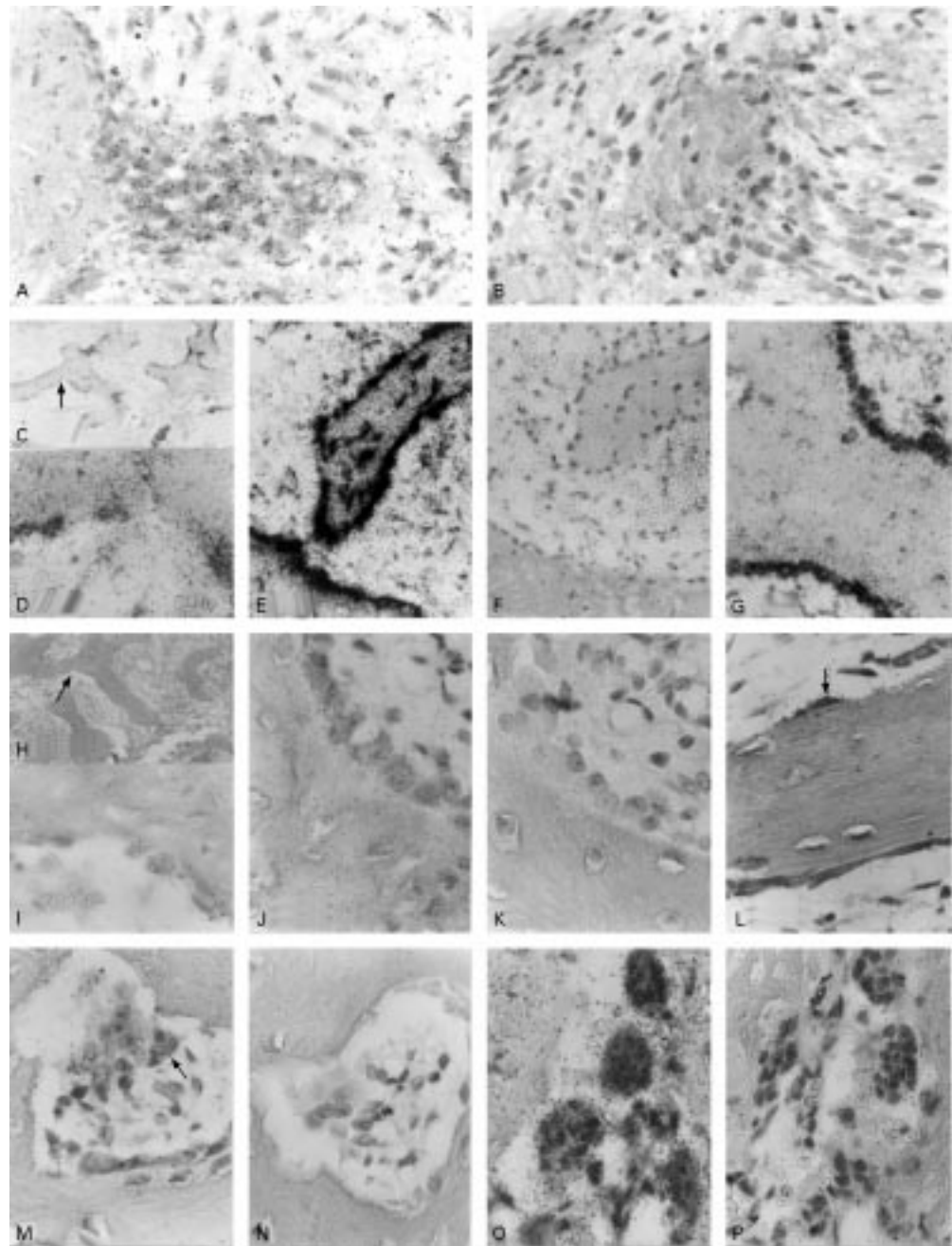


Figure 1 In situ hybridisation (ISH) for matrix gla protein (MGP), osteonectin, osteopontin, and osteocalcin in human fracture callus; haematoxylin and eosin stained. Sections (A) and (B) are from a non-union fracture; sections (C–P) are from normally healing fractures. (A) MGP and (B) control (RNAase): osteoblasts on woven bone surfaces in non-unions were positive for MGP mRNA signal; test and control are from the same area of the same specimen block. Sections (C–G) ISH for osteonectin. (C) In early woven bone osteoblasts were strongly positive for osteonectin mRNA signal; the area selected in (D) shows the area indicated in C (arrow) at a higher magnification; (E) osteonectin and (F) control (RNAase) in early woven bone: non-cuboidal osteoblasts on the surface of woven bone, and many included osteoblasts, were positive for osteonectin mRNA signal; (G) osteonectin in woven bone: plump osteoblasts on the surface of woven bone were positive for osteonectin mRNA signal. Sections (H–N) ISH for osteopontin. (H) In early woven bone osteoblasts were weakly positive for osteopontin mRNA signal, in contrast to a strong osteonectin mRNA signal in the same area (C and D); the area selected in (I) shows the area indicated in H (arrow) at a higher magnification; (J) osteopontin and (K) control (RNAase) in woven bone: plump cuboidal osteoblasts on woven bone were positive for osteopontin mRNA signal, whereas included osteoblasts were negative; (L) osteopontin in lamellar bone: most flattened cells on the surface of lamellar bone were negative for osteopontin mRNA signal, but occasional, scattered cells (about 10% of the total) were positive (arrow); (M) osteopontin and (N) control (RNAase): multinucleate osteoclast-like cells were positive for osteopontin mRNA signal (arrow). Section (O) ISH for osteocalcin and (P) control (RNAase): multinucleate osteoclast-like cells were positive for osteocalcin mRNA signal, to our knowledge a new finding for this cell type.

Table 1 Human: non-collagenous bone matrix protein gene expression in normally healing fractures and non-unions

	MGP	Osteonectin	Osteopontin	Osteocalcin
Haematoma				
Macrophages,	-	+	+	-
Polymorphs	-	-	-	-
Granulation tissue mesenchyme	-	+	+	+
Woven bone osteoblasts	-	+	Early +/- Later +	+
	+ Only in non-unions			
Lamellar bone flat lining cells	-	+/-	+10%, scattered individuals	+/-
Multinucleate resorptive cells	-	-	+	+
Endothelial cells	-	-	-	-

MGP, clone hmGLA-19, ECORI insert 700 bp¹²; osteocalcin, clone hBGP-1, 455 bp, containing a 300 nucleotide (19–318) open reading frame encoding a 100 amino acid human BGP precursor.¹³

PROBE PREPARATION AND HYBRIDISATION

All probes were random prime labelled using the Megaprime labelling system (Amersham, Little Chalfont, UK) to specific activities of $\sim 1 \times 10^8$ counts/min/ μ g using [³⁵S]-*ad*CTP.

The ISH method has been described previously.^{14–18} Briefly, dewaxed sections were rehydrated, pretreated with proteinase K, dehydrated, and air dried; RNAase negative controls were used. Sections were prehybridised for one hour at 37°C in 50% formamide, 1 mg/ml bovine serum albumin, 0.02% (wt/vol) Ficoll, 0.02% (wt/vol) polyvinyl pyrrolidone, 0.6 M NaCl, 0.2 mg/ml sheared salmon sperm DNA, 10 mM Tris (pH 7.4), 0.5 mM EDTA, 10 mM dithiothreitol (DTT), and 10% (wt/vol) dextran sulphate. Hybridisation with heat denatured ³⁵S labelled probe (100 ng/ml prehybridisation mixture) was carried out at 37°C for 16 hours in prehybridisation solution. Aliquots of 50 μ l were applied to each slide and covered with siliconised coverslips. After hybridisation, the tissue sections were washed with a series of high stringency washes: twice for five minutes in 0.5 \times saline sodium citrate (SSC) with 1 mM EDTA and 10 mM DTT; twice for five minutes in 0.5 \times SSC with 1 mM EDTA; four times for five minutes in 0.5 \times SSC at a temperature less than the melting temperature (T_m minus 10°C) for each probe, followed by five minutes at room temperature in 0.5 \times SSC. Sections were then dehydrated and air dried. Autoradiography was performed with K5 emulsion (Ilford, Mobberly, UK), the slides being exposed at 4°C for 10–14 days and then developed in Kodak D-19 developer (Kodak, Paris, France) and counterstained with haematoxylin and eosin.

On microscopy, cells were categorised as chondrocytes, osteoblasts, and so on, in terms of their morphology and relation with their extracellular matrix; that is, the presence of lacunae, capsule/pericellular matrix, processes, and location on the surface of bone trabeculae.

Results

Human fracture callus has a heterogeneous appearance at histological examination, with several of the elements of normal fracture healing being present in close proximity in any one section. These elements include haematoma, fibrous tissue, woven and compact lamellar bone, and cartilage. Because of this heterogeneous appearance, callus specimens were graded 1–3 according to the predominant appearance of the callus, and cellular events were related to the histological grade, as follows: grade 1, fracture blood clot (haematoma) and granulation tissue; grade 2, definitive matrix formation without remodelling of cartilage (grade 2a) and/or bone (grade 2b); grade 3, matrix remodelling. Within the non-union gap, tissues consisted largely of vascularised fibrous tissue or avascular cartilage.

EXPRESSION OF GENES ENCODING

NON-COLLAGENOUS BONE MATRIX PROTEINS

Non-unions: MGP

A population of osteoblasts in non-unions on the surface of woven bone was positive for MGP mRNA signal (fig 1A and B); these were in the zone of new bone formation and in the interface zone. Osteoblasts in the old bone zone were almost always negative, whereas the gap zone rarely contained osteoblasts. Small and large chondrocytes were negative.

Normal fractures: MGP

Signal for MGP mRNA was characteristic of small and large chondrocytes in normal fractures but was never detected over osteoblasts at any location.

Osteonectin, osteopontin, and osteocalcin in normal fractures and non-unions

Scattered macrophages and polymorphs in the haematoma were positive for osteonectin and osteopontin. In the granulation tissue, certain fibrous cells were positive for osteocalcin. (Tissue from these early stages of repair was only available from human fractures that were healing normally; these positive cell types have not been described previously in an animal model of normal fracture healing.⁷) On early woven bone, non-cuboidal osteoblasts were strongly positive

Table 2 Rat: non-collagenous bone matrix protein gene expression in normally healing fracture callus⁷

	MGP	Osteonectin	Osteopontin	Osteocalcin
Day 1	—	Proliferating periosteum	—	—
Day 3	—	Osteoblasts on woven bone	Osteoblasts on woven bone	Osteoblasts on woven bone
Day 5	Immature chondrocytes	Immature chondrocytes		
Day 7		Osteoblastic cells on bone trabeculae (endosteal)	Osteoblastic cells on bone trabeculae (endosteal)	Osteoblastic cells on bone trabeculae (endosteal)
Day 14	Hypertrophic chondrocytes (periosteal)	Osteoblastic cells on bone trabeculae (endosteal)	Osteoblastic cells on bone trabeculae (endosteal) Hypertrophic chondrocytes (periosteal)	Osteoblastic cells on bone trabeculae (endosteal)

for osteonectin mRNA signal, and included osteoblasts were equally positive (fig 1C–F). In later woven bone, cuboidal osteoblasts were strongly positive for osteonectin mRNA signal, but included osteoblasts were only weakly positive (fig 1G). Non-cuboidal osteoblasts on early woven bone were weakly positive for osteopontin mRNA signal (fig 1H and I). Later in repair, cuboidal osteoblasts on the surface of woven bone were moderately positive for osteopontin mRNA signal (fig 1J and K). In contrast to these levels of signal for osteopontin mRNA in the weak to medium range for osteoblasts, a strong signal for osteopontin mRNA was consistent over multinucleate resorptive cells (fig 1M and N). Most flattened cells on the surface of lamellar bone were negative for osteopontin mRNA signal but occasional, scattered cells (about 10% of the total) were positive (fig 1L). Flattened lining cells on lamellar bone were weakly positive for osteonectin and osteocalcin mRNA signals. Multinucleate resorptive cells, in addition to being positive for osteopontin mRNA signal, were also positive for osteocalcin mRNA signal (fig 1M and O), but were negative for osteonectin mRNA signal. Endothelial cells were consistently negative for all non-collagenous bone matrix protein mRNA signals. Tables 1 and 2⁷ summarise the results.

Discussion

Various roles have been proposed for the non-collagenous bone matrix proteins, namely MGP, osteonectin, osteopontin, and osteocalcin, in a number of processes including mineralisation, bone resorption, and cell attachment to extracellular matrix.^{1–19–23} On the basis of findings in MGP deficient mice of inappropriate growth plate calcification, short status, osteopaenia, and fractures, it appears that MGP may be an inhibitor of mineralisation.⁵ Our study examined expression of the genes encoding MGP, osteonectin, osteopontin, and osteocalcin in human fracture healing, a situation of rapid elaboration of intramembranous and endochondral bony matrix. Gene expression of these molecules during the process of fracture healing has been investigated previously in a rat model of normal fracture healing (osteonectin and osteocalcin by means of northern blotting⁶; osteocalcin by means of ISH⁸; and MGP, osteonectin, osteopontin, and osteocalcin by means of ISH.⁷)

Our studies on fracture callus of normally healing fractures have shown an osteoblast phenotype that has not been reported previously in humans. The osteoblast phenotype in an animal model of normal fracture healing was MGP negative, but osteonectin, osteopontin, and osteocalcin positive (table 2).⁷ Normal fracture healing in humans is consistent with these findings, except for low levels of osteopontin gene expression (compare with osteoclasts²⁴; fig 1I, J, and M), which might be related to the slower healing rates seen in humans compared with the rat. Also notable was osteopontin gene expression by occasional, scattered, flattened lining osteoblasts (fig 1L), in contrast to relative inactivity of the residual

population; this may relate to a signalling role for osteopontin.²⁵

In non-unions, the osteoblast phenotype was different. In particular, osteoblasts on woven bone expressed the gene for MGP. Expression of this gene, characteristic of chondrogenic tissues during skeletogenesis, has previously been reported in cultured osteoblasts^{26–31} but, to our knowledge, not in osteoblasts in skeletal samples.^{4–5} Expression of the MGP and collagen type III¹⁸ genes by osteoblasts in biopsies of non-unions raises fundamental questions about control mechanisms operating in osteoblasts, and could have important implications for understanding mechanisms leading to fracture non-union.

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