Expression of osteopontin mRNA by osteoclasts and osteoblasts in modelling adult human bone

Karen Merry*, Robert Dodds†, Amanda Littlewood‡ and Maxine Gowen†

Bath Institute for Rheumatic Diseases and the University of Bath, Bath

*Present address: CELLTECH Ltd, 216 Bath Road, Slough SL1 4EN, Berks, UK
†Present address for correspondence: SmithKline Beecham Pharmaceuticals, Department of Cellular Biochemistry, Units of Bone Biology and Immunology, King of Prussia, PA 19406, USA
‡Present address: Kantonspital, CH-4031 Basel, Switzerland

INTRODUCTION

Bone matrix is a highly organised and complex tissue. It consists of an inorganic component in the form of hydroxyapatite and an organic component consisting of proteins and proteoglycans (Franzen and Heinegard, 1984). In addition to the type 1 collagen framework other non-collagenous proteins are present, including the phosphoprotein osteopontin, originally named bone sialoprotein I as it contains many sialic acid moieties. The amount of osteopontin present in bone matrix is species dependent, but is estimated at approximately 2% of non-collagenous proteins (Franzen and Heinegard, 1985).

Osteopontin binds tightly to hydroxyapatite in bone, presumably via an acid-rich stretch of nine aspartic acid residues. It appears to form an integral part of the mineralized matrix since it can be extracted only under denaturing conditions (Fisher et al., 1987). Sequence analysis of the osteopontin gene reveals that it contains an RGD tripeptide sequence and therefore may interact with integrin receptors (Kiefer et al., 1989). It has been postulated to be a ligand for the vitronectin (αvβ3) receptor (Reinholt et al., 1990) and this suggests a possible role of osteopontin in osteoclast attachment and function. Osteopontin is not a specific bone protein as its mRNA can be detected in high levels in skin and kidney, although the kidney form may be genetically distinct (Gotoh et al., 1991). Neuronal cells in the brain and inner ear have also been demonstrated to express high levels of osteopontin mRNA. The whole population of osteoclasts in the osteoclastoma tissue expressed high levels of osteopontin mRNA, indicating that expression is not restricted to osteoclasts involved in bone resorption.

SUMMARY

Over recent years several non-collagenous matrix proteins of bone have been isolated and characterized. One of these proteins, osteopontin, has been shown to be synthesized by osteoblasts and deposited in the bone matrix where it is thought to bind to hydroxyapatite. However much of the functional evidence is circumstantial, and the precise function of osteopontin has not been fully elucidated.

We have used in situ hybridization techniques to investigate the expression of osteopontin mRNA in a variety of human bone tissues. Cryostat sections of human osteophyte and osteoclastoma tissue were hybridized with an antisense RNA probe for osteopontin. Sense transcripts were used as a negative control to assess non-specific binding.

There was a very distinct pattern of osteopontin mRNA expression in these tissues. Plump osteoblasts adjacent to the osteoid matrix expressed high levels of osteopontin mRNA, whilst flattened osteoblasts demonstrated weaker expression. The most striking feature of osteopontin mRNA expression was the high levels detected in osteoclasts. Osteoclasts in resorption lacunae and those distant from resorption sites both expressed osteopontin mRNA, suggesting that attachment was not a prerequisite for osteopontin expression. A population of mononuclear cells in resorption lacunae was also observed to express high levels of osteopontin mRNA. The whole population of osteoclasts in the osteoclastoma tissue expressed high levels of osteopontin mRNA, indicating that expression is not restricted to osteoclasts involved in bone resorption.

This study confirms that human osteoblasts are capable of synthesizing osteopontin. However it highlights the fact that osteoclasts may not be dependent on osteopontin present in the matrix for attachment, as they are capable of synthesizing their own osteopontin to facilitate anchorage to the bone matrix prior to resorption.

Key words: osteopontin, osteoblast, osteoclast, bone
eralized matrix (Reinholt et al., 1990) particularly in regions of the bone surface where osteoclasts are anchored. It may act, therefore, as a means of attachment for these cells to the bone surface (Reinholt et al., 1990).

Osteoblasts obtained from many species have been shown to be capable of synthesizing osteopontin in vitro, and include osteoblasts isolated from fetal rat calvariae and the rat cell line ROS 17/2.8 (Prince et al., 1987). Osteopontin is also synthesized by chondrocytes (Castagnola et al., 1991), although its expression seems to be restricted to areas where the cartilage is being remodeled into bone. Synthesis of osteopontin by normal human bone cells in culture has not been documented, but it has been suggested that it is not a major secretory product of these cells (Gehron Robey, 1989). Osteopontin may also be important in cell growth as it can be shown to promote collagen independent cell spreading and attachment of ROS17/2.8 cells and normal human osteoblasts in culture (Gehron Robey, 1989).

Despite extensive studies on osteopontin in several species, its precise function in bone formation and remodeling remains to be fully elucidated. We have used in situ hybridization of RNA probes for osteopontin to cryostat sections of human osteophytic bone and osteoclastoma tissue to investigate its expression at different stages of bone development. Human osteophyte is a rapidly developing bone tissue and a major characteristic of osteoarthritic joints. This tissue has proved to be a good model tissue for the study of human bone development and remodeling (Dodds et al., 1990b). Using this approach we aim to define which cell types are capable of expressing osteopontin mRNA, and characterise in which bone microenvironments the transcription of this protein is initiated.

**MATERIALS AND METHODS**

**Preparation of tissue**

Developing osteophytes were dissected from the femoral heads removed from patients undergoing hip arthroplasty for osteoarthritis. Osteoclastoma tissue was obtained as curettage samples from patients undergoing hip arthroplasty for osteoarthritis. Osteoclastoma tissue was obtained as curettage samples from patients undergoing hip arthroplasty for osteoarthritis.

Histological evaluation of the developing osteophyte was carried out using a modified Romanowsky Wright’s stain (Fast green in methanol, Eosin G and Thiazine Dye/Methylene blue). Briefly, the staining patterns in bone and cartilage tissue are as follows: mineralized matrix remains unstained (white appearance) whereas the various unmineralized matrices (cartilage, osteoid, fibrous tissue) stain in a range from pink (e.g. osteoid) to purple/blue (e.g. mature cartilage). Nuclei stain purple and the cytoplasm stains pink - weakly blue - dark blue (highly biosynthetic cell).

Cross and transverse sections of osteophyte and surrounding tissue were cut, starting at the tip of the outgrowth and proceeding downwards in the direction of the subchondral bone to disclose the various stages of maturation. Further details of the histological findings, specifically pinpointing important aspects in bone cell biology, are presented in the legends to the figures.

**In situ hybridization**

**Pretreatment of sections**

Small pieces of osteophyte were mounted on cryostat chucks. Sections were cut and transferred to TESPA (3-aminopropyl triethoxy silane)-coated slides and then fixed in 4% paraformaldehyde (pH 7.2), for 5 minutes, washed in PBS and air dried. The sections could then be stored desiccated for up to one month at −20°C. Following rehydration with PBS, the sections were treated with 0.1 M glycine/PBS for 2×3 minutes then PBS for 3 minutes. The sections were then decalcified using 0.25 M sodium citrate buffer (pH 2.5) for 1 hour. To decrease nonspecific binding of the RNA probes to structural proteins the sections were treated with protease K, 1 µg/ml diluted in 0.1 M Tris (pH 8.0) 50 mM EDTA (pH 8.0) at 25°C for 20 minutes. To maintain the cellular localization of the mRNA the sections were then refixed in 4% paraformaldehyde. Following rehydration in PBS for 2×3 minutes the remaining proteins were acetylated using acetic anhydride (0.25% in 0.1 M triethanolamine) for 10 minutes at room temperature. Finally a prehybridization step was performed in 50% deionized formamide, 2×SSC for 10 minutes.

**Hybridization**

The osteopontin cDNA template was prepared from the plasmid pOP/10, which was a kind gift from Dr Marian Young, Bethesda, MD, USA. RNA probes were constructed using the Boehringer Mannheim SP6, T7 in vitro transcription kit with [35S]thioCTP. Typically 2 µg of linear template were used, and yielded RNA transcripts with a specific activity in excess of 108 cpm/µg. The template DNA was removed using 20 units of RNase-free DNase at 37°C for 15 minutes. The unincorporated nucleotides were removed by separation on a spinning Sephadex G50 column. The RNA probes were then stored at −20°C until used.

The sections were prehybridized in a solution containing 50% formamide, 2×SSC, 10% dextran sulphate, 10 mM DTT, 10 units/ml RNase inhibitor, 5 µg/ml tRNA, 1×Denhardt’s and 200 µg/ml ssDNA in a humidified chamber for 3 hours at 42°C, prior to the addition of the RNA probe. Approximately 2 ng of RNA probe with a specific activity of 1×108 cpm/µg was added to each section and hybridized overnight at 42°C.

**Post-hybridization washing**

The coverslips were removed by gently agitation the sections in 4×SSC. The slides were then washed in 4×SSC at 37°C in a shaking waterbath for 4×15 minutes, followed by a wash in 10 mM Tris/HCl, 0.5 M NaCl, pH 8.0 for 10 min. The sections were then treated with 20 µg/ml RNase A in 10 mM Tris/HCl, 0.5 M NaCl, pH 8.0 at 37°C for 30 minutes, to remove any nonspecific hybridization. More stringent washes, in 2×SSC for 30 min at 37°C and 0.1×SSC for 30 min at 37°C were then performed to remove any residual nonspecific binding. Finally, the slides were dehydrated through a 70%, 80%, 95%, 100% ethanol series containing 300 mM ammonium acetate and air dried. The slides were then coated in Amersham LM-1 emulsion under a red safe light and exposed at 4°C for 2 weeks. The slides were developed using Ilford Phenisol developer and counterstained with Wright’s stain to highlight tissue morphology. The expression of osteopontin was analysed in twelve osteophytes and four different osteoclastomas, in four separate experiments. The extent of the hybridization signal was assessed by the autoradiographic grain density over the cell.
RESULTS

Osteophyte formation
The type of osteophytes described in this study develop in the dense mesenchymal connective tissue that forms on the denuded surfaces of the osteoarthritic head and growth develops by the processes of endochondral and intramembranous bone formation (Figs 1a and 2a). Mesenchymal cells differentiate into hypertrophic chondrocytes either directly or by way of a fibrocartilage transition stage. The

Fig. 1. Endochondral bone formation in the developing osteophyte. Serial sections were (a) stained with Wright’s stain (b) hybridized with osteopontin mRNA antisense probe and (c) hybridized with osteopontin sense probe (control). Sections b and c were counterstained with methylene blue. No background signal was observed in the sense control section (c). Key: chondroblasts differentiating from the mesenchymal cells of the surrounding connective tissue (*); hypertrophic chondrocytes (C); chondrocytes within the calcified cartilage (large arrowhead); osteoblasts (small arrowheads) loosely apposed to the calcified cartilage surfaces (no osteoid) or forming osteoid (arrows). ×10.

Fig. 2. Intramembranous bone formation in the developing osteophyte. Serial sections were (a) stained with Wright’s stain (b) hybridized with osteopontin mRNA antisense probe and (c) hybridized with osteopontin sense probe (control). Sections b and c were counterstained with methylene blue. Key: osteoblasts differentiating from the mesenchymal connective tissue cells and secreting osteoid (arrowheads); undifferentiated mesenchymal cells (*); encased osteoid osteocytes (o); osteoblasts apposed to trabecular bone (arrow; no obvious osteoid formation was evident). No background signal was observed in the sense control section (c). ×10.
cartilage is remodeled into trabecular bone by the process of endochondral bone formation. At precise locations the mesenchymal cells differentiate into osteoblasts and lay down woven bone on residual cartilage (periosteal-like intramembranous bone formation; Fig. 2a) and in areas not associated with cartilage (true intramembranous bone formation). This bone develops into the cortical shaft of the osteophyte.

Osteopontin expression was detected in chondrocytes, osteoblasts and osteoclasts (Table 1). The level of expression (hybridization signal was judged by grain density) was dependent on the developmental stage of the growing osteophyte, and the stage of differentiation of each cell type.

Table 1. Osteopontin mRNA expression in human bone and osteoclastoma tissue

<table>
<thead>
<tr>
<th>Osteopontin expression</th>
<th>Cartilage</th>
<th>Connective tissue cells</th>
<th>Chondroblasts</th>
<th>Fibrocartilage cells</th>
<th>Hypertrophic chondrocytes</th>
<th>Chondrocytes within calcified cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone formation</td>
<td>Pre-osteoblasts</td>
<td>+++</td>
<td>Osteoblasts forming intramembranous bone</td>
<td>+++</td>
<td>Osteoblasts forming trabecular bone</td>
<td>++</td>
</tr>
<tr>
<td>Bone resorption</td>
<td>Osteoclasts</td>
<td>±</td>
<td>Osteoclasts resorbing bone</td>
<td>++++</td>
<td>Mononuclear cells in lacunae</td>
<td>+++</td>
</tr>
<tr>
<td>Osteoclastoma</td>
<td>Osteoclasts</td>
<td>++++</td>
<td>Mononuclear stromal cells</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The extent of the hybridization signal (no signal to high signal; − to ++++) was assessed by the autoradiographic grain density over the cell.

Cartilage formation

The primary phase of osteophyte development involves the development of cartilage within the mesenchymal connective tissue and the subsequent calcification of this cartilage. Fibrocartilage cells and mesenchymal cells, differentiating into early hypertrophic chondrocytes at the periphery of the osteophyte, expressed high levels of osteopontin mRNA (Fig. 1). Lower levels of expression were detected in mature hypertrophic chondrocytes (Fig. 1), but in areas of calcifying cartilage no expression was detected (Fig. 1).

Bone formation

Following osteoclastic and mononuclear resorption of the cartilage, osteoblasts secrete woven bone onto the cartilage remnants (endochondral bone formation) and trabeculae of woven bone thus replace the forming cartilage. Osteoblasts laying down woven osteoid expressed high levels of osteopontin mRNA (Fig. 1).

Osteoblasts differentiating from the surrounding connective tissue and secreting osteoid at the periphery of the developing osteophyte expressed extremely high levels of osteopontin mRNA (Fig. 2).

With the progression from cuboidal active osteoblasts...
Fig. 4. Osteopontin mRNA expression in chondroclasts and osteoclasts within the developing osteophyte. (a) Chondroclasts at a site of cartilage resorption (arrowheads). (b) Clusters of osteoclasts at a site of woven bone resorption. (c) Serial section hybridized with osteopontin sense probe (control) showed negligible background staining. (d) Osteoclast (arrow; Wright’s stain) distant from the bone surface. (e) Serial section hybridized with osteopontin antisense probe (arrow). Thin elongated osteoblasts (inactive; arrowheads) demonstrated low osteopontin expression compared to plump osteoblasts (large arrow). (f) Serial section hybridized with osteopontin sense probe (control) showed no nonspecific background grains. (g) Osteopontin mRNA expression in an osteoclast resorbing lamellar bone; only the occasional osteoblast demonstrated observable hybridization signal (arrow). (h) Serial section hybridized with osteopontin sense probe shows a low, diffuse signal (large arrow) that contrasts with the intense signal localized to the osteoclasts with the antisense probe. ×20 (a-f), ×40 (g and h).
apposed to a thin strip of osteoid) to flatter and more oblong cells (intermediate osteoblasts; thick osteoid/osteocyte encasement) osteopontin expression decreased markedly (Fig. 3). Thinner more elongated cells (inactive osteoblasts morphologically indistinguishable from the flat lining cells which cover quiescent surfaces; see later on in Fig. 4d,e and f) demonstrated low osteopontin expression.

Distinct populations of plump osteoblasts in lamellar bone expressed high levels of osteopontin (Fig. 3c), however, the majority of cells demonstrated little osteopontin expression (see for example Fig. 4f).

**Osteoclast resorption**

During the initial stages of cartilage resorption, and the transition of calcifying cartilage to woven bone, osteopontin expression was low or not detectable in osteoclasts (chondroclasts; Fig 4a). In contrast, at sites of active woven bone
remodeling, clusters of osteoclasts were observed to express very high levels of osteopontin mRNA (Fig. 4b and c). Osteopontin expression was not restricted to resorbing osteoclasts, as it was also detected in osteoclasts distant from the bone surface (Fig. 4d,e and f). Occasional osteoclasts resorbing lamellar bone demonstrated a similarly high osteopontin expression (Fig. 4g and h). The entire population of osteoclasts in the osteoclastoma tissue (Fig. 5) demonstrated high osteopontin mRNA expression; expression was also detected in the mononuclear cells within the osteoclastoma tissue.

Within completed resorption sites of osteophytic bone, mononuclear cells, possibly macrophages or preosteoclasts, expressed high levels of osteopontin (Fig. 6).

**DISCUSSION**

Osteopontin is not a specific bone protein; its mRNA has been demonstrated in skin, kidney (Gotoh et al., 1991) and neuronal cells in the brain and inner ear (Nomura et al., 1988). We have further demonstrated expression of osteopontin mRNA in a variety of cell types in human bone and cartilage as summarized in Table 1. The most surprising feature of osteopontin expression was the large amounts of mRNA detected in certain populations of osteoclasts, as it had been previously assumed that osteoblasts were the major synthetic cells for osteopontin.

Since osteopontin is postulated to act as anchor for osteoclasts to bone (Reinholt et al., 1990), these results suggest that osteoclasts may facilitate, in certain circumstances, their own attachment to bone. Osteopontin expression was not dependent on attachment to bone, since high levels of osteopontin mRNA were detected in many osteoclasts distant from the bone surface, and the entire population of osteoclasts in the osteoclastoma tissue expressed osteopontin mRNA. Recently Bianco et al. (1991) demonstrated that osteoclasts in human fetal bone expressed both bone sialoprotein II (BSP) mRNA and protein. BSP is also thought to be a cell attachment factor. It is possible that osteoclasts express these two sialoproteins so that they are not limited to sites of previous osteoblastic activity for attachment.

It is interesting to note that certain populations of chondrocytes expressed osteopontin. The expression decreased as the chondrocytes matured; differentiating mesenchymal cells (chondroblasts) expressed the highest level of osteopontin mRNA, hypertrophic chondrocytes expressed lower levels and chondrocytes in calcifying cartilage expressed no osteopontin mRNA. The role of osteopontin in cartilage is unknown. The expression of osteopontin mRNA by chondrocytes could illustrate a role for these cells in synthesizing matrix that is thus primed for osteoclastic resorption and remodeling. Alternatively, this synthetic role for chondrocytes may reflect the fact that chondrocytes and osteoblasts arise from a common precursor and may share the expression of some matrix proteins. The expression of osteopontin by osteoblasts was also dependent on the maturation stage of the cell. Osteoblasts actively secreting osteoid expressed high levels of osteopontin. In contrast, quiescent lining cells demonstrated very little expression.

This expression profile is similar to many matrix components including osteonectin (Bianco et al., 1988).

It is possible that some of the non-collagenous proteins of bone may mediate cell:cell interactions instead of purely cell:matrix adhesion. For instance, osteopontin may facilitate cell:cell interactions via binding to the integrins on the cell surface. It could therefore play a role in preosteoclast fusion.

The expression of osteopontin in certain mononuclear cells in resorption lacunae was unexpected and it is tempting to speculate on the origin and function of these cells: macrophages or preosteoclasts. It is possible that their expression of osteopontin is a ‘post-resorptive’ signal to recruit osteoblasts to initiate bone formation in the resorption lacunae. Preliminary results have indicated that macrophages and macrophage polykaryons within inflammatory osteophtic synovium express high levels of osteopontin mRNA.

The expression of bone matrix proteins such as osteopontin by osteoblasts highlights the potential of this cell type to synthesize products other than those directly involved with the dissolution of the matrix. In this case the osteopontin may be involved with the adhesion of the osteoclast itself to the bone matrix, or it may be secreted onto the bone surface to act as a signaling molecule to other cell types.

**REFERENCES**


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