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Distribution of matrix metalloproteinases and their inhibitor, TIMP-1, in developing human osteophytic bone

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ABSTRACT

Connective tissues synthesise and secrete a family of matrix metalloproteinases (MMPs) which are capable of degrading most components of the extracellular matrix. Animal studies suggest that the MMPs play a role in bone turnover. Using specific polyclonal antisera, immunohistochemistry was used to determine the patterns of synthesis and distribution of collagenase (MMP-1), stromelysin (MMP-3), gelatinase A (MMP-2) and gelatinase B (MMP-9) and of the tissue inhibitor of metalloproteinases-1 (TIMP-1) within developing human osteophytic bone. The different MMPs and TIMP showed distinct patterns of localisation. Collagenase expression was seen at sites of vascular invasion, in osteoblasts synthesising new matrix and in some osteoclasts at sites of resorption. Chondrocytes demonstrated variable levels of collagenase and stromelysin expression throughout the proliferative and hypertrophic regions, stromelysin showing both cell-associated and strong matrix staining. Intense gelatinase B expression was observed at sites of bone resorption in osteoclasts and mononuclear cells. Gelatinase A was only weakly expressed in the fibrocartilage adjacent to areas of endochondral ossification. There was widespread but variable expression of TIMP-1 throughout the fibrous tissue, cartilage and bone. These results indicate that MMPs play a role in the development of human bone from cartilage and fibrous tissue and are likely to have multiple functions.

Key words: Collagenase; gelatinase; stromelysin; osteoblasts; osteoclasts.

INTRODUCTION

Skeletal development and homeostasis depend on the precise regulation of resorbing and bone forming events, including the degradation and removal of constituents of the extracellular matrix and the synthesis and deposition of new components. Matrix metalloproteinases (MMPs) are capable of degrading all components of connective tissue at physiological pH (Murphy & Reynolds, 1993) and are likely to be involved in bone matrix degradation (Everts et al. 1992). MMPs are a family of zinc-dependent proteinases that include the collagenases (MMPs 1, 8, 13), which degrade interstitial collagens types I, II and III into three-fourths and one-fourth fragments, the gelatinases (MMPs 2, 9), which degrade type IV collagen and gelatin, and the stromelysins (MMPs 3, 10), which are more general proteinases that cleave proteoglycans, fibronectin, laminins, gelatin and casein. All MMPs are secreted from the cell as latent enzymes and become activated in the pericellular environment by disruption of the Zn^{2+}-cysteine bond which blocks the reactivity of the active site. For collagenase and stromelysin this activation process may involve plasmin, whilst stromelysin is also capable of activating collagenase (Murphy et al. 1992). The action of MMPs is further regulated by a family of 4 specific inhibitors TIMP-1 to 4 (tissue inhibitor of matrix metalloproteinase). The most widely distributed and studied is TIMP-1, a 28 kDa glycoprotein synthesised by most connective tissue cells (Cawston, 1986).

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Animal studies have shown that MMPs are involved in endochondral ossification (Dean et al. 1989; Breckon et al. 1995) and osteoclastic degradation of bone matrix (Raina, 1972; Delaissé et al. 1988; Everts et al. 1992). Osteoblasts secrete collagenase in response to osteotropic agents (Heath et al. 1984; Meikle et al. 1991) and collagenase has also been identified in the subosteoclastic compartment of bone undergoing resorption (Delaissé et al. 1993). Collagenase, stromelysin, gelatinase B and TIMP have been immunolocalised in isolated rabbit osteoclasts (Hill et al. 1994), PTH-induced collagenase and gelatinase B mRNA production in rat bones has also been reported (Witty et al. 1996). Wucherpfennig et al. (1994) and Rao et al. (1995) have described the presence of mRNA for gelatinase B in giant cells of osteoclastoma tissue. We have reported the constitutive production of gelatinase A and TIMP and stimulated synthesis of collagenase, stromelysin and gelatinase B by human osteoblasts in vitro (Meikle et al. 1992) and have also demonstrated the presence of collagenase in osteoblasts and osteoclasts in human bone in vivo (Bord et al. 1996).

Osteophytes are bony and cartilaginous outgrowths which form at the margin of osteoarthritic large joints. Their rapid growth, with endochondral and intramembranous bone formation occurring concurrently and their growth pattern, which mimics changes seen in the epiphyseal growth plate of growing long bones, provides an ideal model to study events involved in human bone growth in vivo.

The aim of the present investigation was to extend our previous observations (Bord et al. 1996) by investigating the distribution of collagenase-1 (MMP-1), gelatinase A (MMP-2), gelatinase B (MMP-9), stromelysin-1 (MMP-3) and TIMP in developing bone, using the human osteophyte as a model for rapidly developing human bone.

MATERIALS AND METHODS

Tissue

Ten osteophytes removed from shoulder and hip joints after routine joint replacement surgery were used for the study. Informed written consent was obtained and approval was obtained from the local ethics committee. The orientation of the osteophyte in relation to the joint was noted and the bone cut into suitable sized pieces (1 cm^3). Following immersion in 5% polyvinyl alcohol (PVA) for 1 min they were snap frozen in liquid nitrogen and stored at -70 °C. Frozen sections were obtained using a Bright cryostat (Huntingdon, England) with a cabinet temperature of -30 °C, equipped with a slow drive high torque motor with automatic speed control and a highly polished tungsten carbide knife. Sets of serial sections (9 μm) were cut at 125 μm intervals throughout the osteophytes, picked off onto glass slides coated with 2% APES (3-aminopropyltriethoxy-silane), air dried for 10 min, fixed in 4% paraformaldehyde for 22 min at room temperature and washed in phosphate buffered saline (PBS).

Histology

Undecalcified unfixed 9 μm frozen sections were stained with Diff-Quik (Baxter Dade AG) for general morphology, with von Kossa with a Van Giesen counterstain to assess matrix mineralisation, and with safranin O or toluidine blue to determine proteoglycan content. Alkaline phosphatase staining to identify osteoblast populations was detected using a coupled reaction with α-naphthyl acid phosphate and Fast Red TR (Bradbeer et al. 1994). Tartrate-resistant acid phosphatase (TRAP) staining in osteoclasts and preosteoclasts was demonstrated by reactivity with AS-BI phosphate and sodium tartrate, postcoupled with Fast Garnet (Loveridge et al. 1991).

Antibodies

Well characterised specific polyclonal antibodies to human gelatinase A (Hipps et al. 1991), gelatinase B (Murphy et al. 1989), human stromelysin (Allan et al. 1991) and human TIMP-1 (Hembry et al. 1985) were raised in sheep. Human interstitial collagenase was purified according to Whitham et al. (1986), injected into sheep and the resulting antiserum characterised by Western blotting, inhibition and immunolocalisation. Specificity was also confirmed by positive staining on immunolocalisation of NSO mouse myeloma cells transfected with the human collagenase gene (Murphy et al. 1992) and absence of staining of cells transfected with either human stromelysin-1 and 2 or gelatinase A or B. This antiserum has been successfully used to immunolocalise collagenase in human synovium and bone (Hembry et al. 1995; Bord et al. 1996) and does not react with human neutrophil collagenase. Immunoglobulins for all antisera were prepared by triple ammonium sulphate precipitation. Pooled normal sheep serum IgG (NSS) was used as a control. The secondary antibody was a biotinylated rabbit antisheep IgG (Vector Laboratories).

Immunohistochemistry

Immunolocalisation was carried out using an indirect immunoperoxidase system. Endogenous peroxidase and nonspecific binding were blocked by incubation with ImmunoPure peroxidase suppressor (Pierce, 22 min) and extensive washing followed by 20% normal rabbit serum (12 min) before the primary antibody incubation (overnight at 4 °C in a humid chamber). Following further washing the second antibody was applied (5 μm / ml, 40 min at room temperature). The sections were washed and the signal amplified using avidin biotin complex (ABC, Vector Laboratories, 30 min at room temperature). Sites of antigen binding were visualised using 3,3′-diaminobenzidine (DAB) or 3-amino-ethylcarbazole (AEC) as chromogens. Some sections were lightly counterstained with haematoxylin or methyl green to detect nuclei. Sections were mounted in aqueous mount (90% glycerol in PBS) and observed under bright field microscopy on an Olympus BH-2 microscope. Photographs were taken using Ektachrome 64 film.

Quantitation

Cells within specific areas were examined for staining. These areas included the fibrous tissue, defined morphologically as connective tissue containing elongated fibroblast-like cells and vascular cells; cartilage and endochondral bone formation with associated chondrocytes, vascular cells and osteoblasts; intramembranous ossification with associated fibroblasts and osteoblasts; and cells associated with bone modelling and remodelling.

For each cell type, the percentage of cells staining was assessed quantitatively by counting 5 fields from each section and the results expressed as the number of cells staining positively as a percentage of the total cell number. These figures were tabulated as follows: no staining scored as $-$; less than 20% of cells staining positively scored $+$; 20–50% positive cells as $++$ and more than 50% of cells exhibiting positive staining scored $+++$. The presence of matrix staining was also noted.

RESULTS

Histological features of osteophytic bone

The osteophytes were variable in size and shape, but exhibited similar histological features to those reported by others (Dodds & Gowen, 1994; Resnick & Niwayama, 1995). The orientation of the osteophytes was noted at time of surgical removal and the cutting of sets of 9 μm serial sections every 125 μm throughout some osteophytes made it possible to follow the sequence of events in their growth. Occasionally newly forming 'miniosteophytes' at the peripheral edge of the main osteophyte were observed (Fig. 1). These appeared to develop from small areas of fibrous tissue with a toluidine blue and safranin O-negative matrix containing flattened fibroblast-like cells (Fig. 1*A*). Vascular invasion was associated with the appearance of a narrow fibrocartilage outer collar, containing collagenous fibres with cartilage cells and scant cartilage matrix. This, together with an outer layer of fibrous tissue, surrounded the osteophyte. A central area with bone formation and resorption was evident, with mineralised bone and thick osteoid seams (Fig. 1*B*, *C*, *D*). As the 'miniosteophyte' enlarged it became encompassed within the fibrocartilage collar surrounding the main osteophyte.

Both endochondral and intramembranous bone formation were evident, as were woven and mature lamellar bone. Within the fibrous tissue, elongated fibroblast-like cells were seen adjacent to discrete islands of cartilage containing differentiating chondrocytes. Endochondral ossification was evident with deposition of woven bone by osteoblasts on the resorbed surfaces (Fig. 1*E*). Areas of cartilage contained clusters of highly vacuolated chondrocytes surrounded by strong toluidine blue or safranin O positive staining matrix, indicating a high proteoglycan content (Fig. 1*F*). In some areas, bone appeared to be deposited directly in the fibrous tissue and in bone marrow spaces, consistent with intramembranous bone formation. Large populations of osteoblasts were observed in the marrow spaces adjacent to areas of woven or lamellar bone, on which thick osteoid seams were often present.

TRAP and alkaline phosphatase activity

Intense TRAP activity was observed at the surfaces of the resorbed calcified cartilage and in hypertrophic chondrocytes. Osteoclasts and mononuclear cells associated with resorption of bone matrix at sites of intramembranous and endochondral ossification also stained strongly (Fig. 1*G*). In areas of bone formation, where thick osteoid seams and layers of plump osteoblasts were observed, there was intense staining for alkaline phosphatase. Osteoblasts at sites of new bone formation demonstrated alkaline phosphatase positivity (Fig. 1*H*), whilst osteoclasts and mononuclear cells were negative.

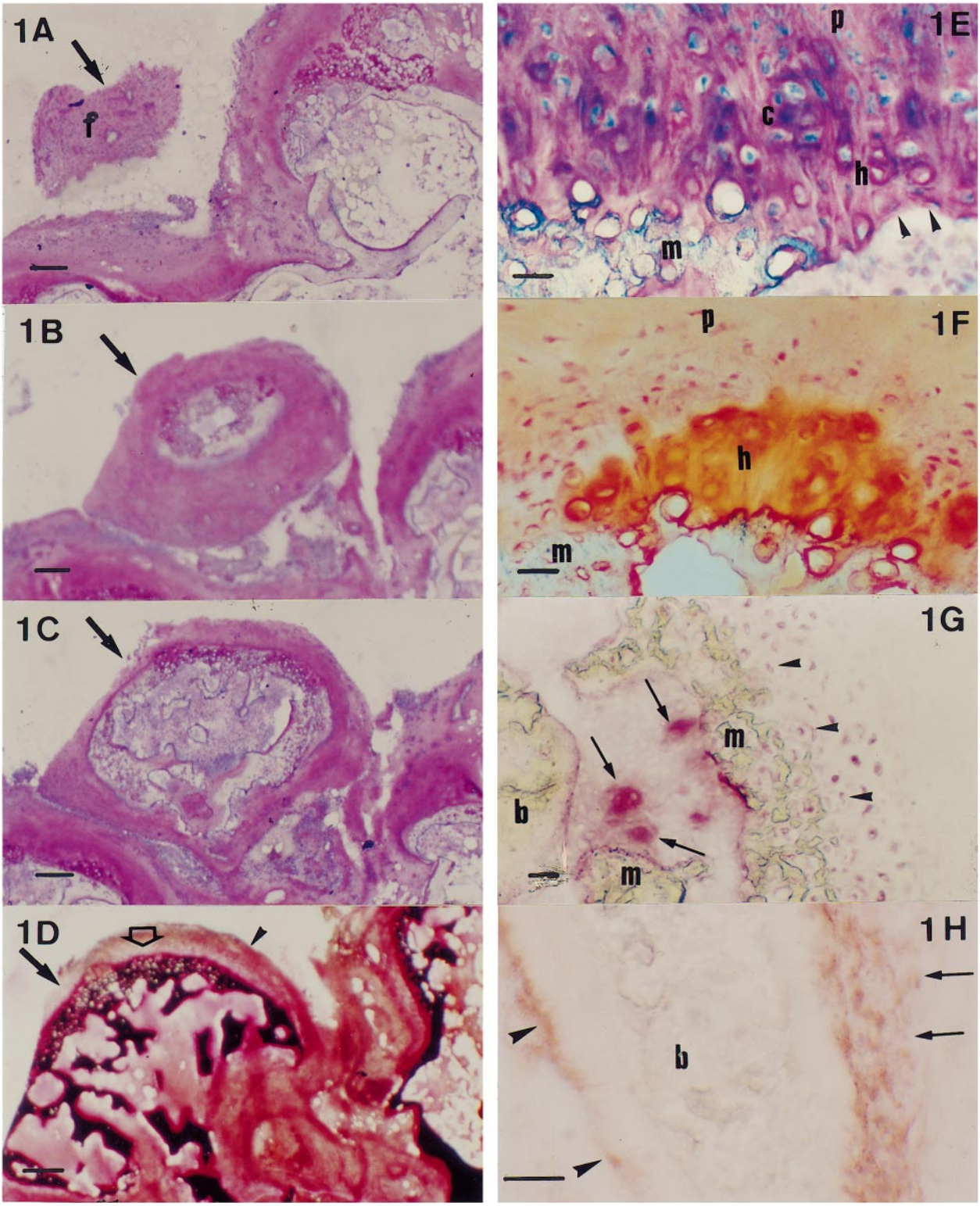

Fig. 1. Histology of sections of osteophytic bone. (*A–C*) Sections cut 125 μm apart stained with Diff-Quik show the development of a 'miniosteophyte' (arrow). (*A*) Fibrous tissue (f) is situated at the periphery of the main body of the osteophyte. (*B*) Rapid development of (*A*) has led to the formation of a fibrocartilage outer collar with a bone forming / resorbing centre. (*C*) Further remodelling gives rise to the formation of a trabecular structure and the incorporation of this 'miniosteophyte' into the main osteophyte. (*D*) Serial section to (*C*) stained with von Kossa shows the areas of mineralised bone (black colour). The fibrous layer (arrowhead) and fibrocartilage collar (open arrow) surround the cartilage and bone. (*E*) Diff-Quik stained section shows an area of endochondral ossification. Areas of resorption of the cartilage (arrowheads) are adjacent to mineralised bone (m), with proliferative (p), mature (c) and hypertrophic (h) chondrocytes. (*F*) Safranin O stained section shows endochondral bone formation with areas of high proteoglycan content in the hypertrophic cartilage indicated by the intense orange staining. (*G*) High levels of TRAP activity shown in osteoclasts (arrows), adjacent to the mineralising bone (m) and in hypertrophic chondrocytes (arrowheads). b, bone. (*H*) Alkaline phosphatase activity in multilayers of osteoblasts on one bone forming surface (arrows) and in a single layer of osteoblasts on the opposite surface (arrowheads). b, bone. Bars: A–D, G, 100 μm; E, F, H, 20 μm.

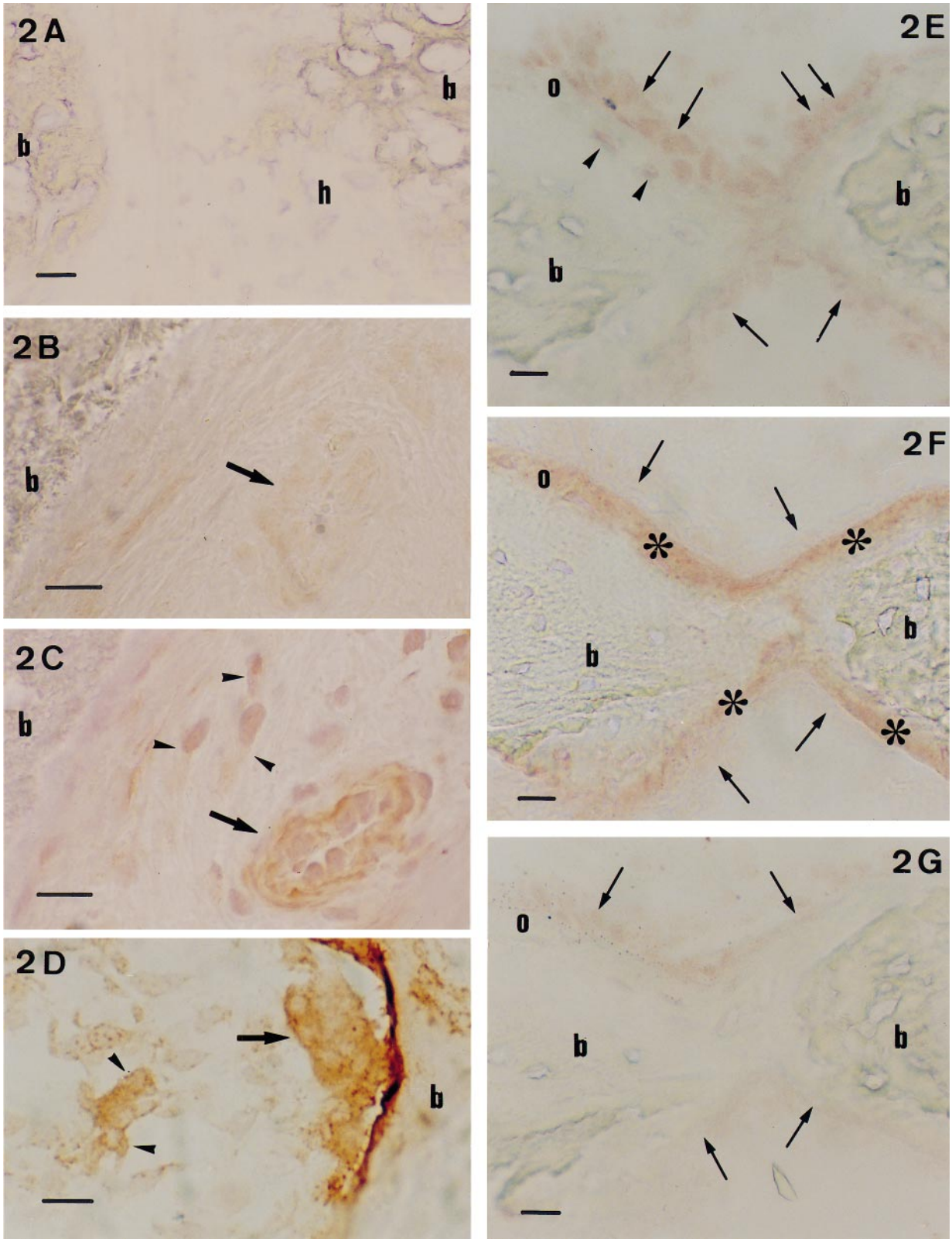

Fig. 2. Immunoperoxidase localisation studies using sheep polyclonal antibodies. (*A*) No detectable signal is seen in the normal sheep serum control in an area of endochondral ossification. (*B*) A normal sheep serum control of bone, a blood vessel (arrow) and marrow space shows absence of staining whilst in a serial section (*C*), collagenase expression is clearly evident in the blood vessel (arrow) and in some adjacent cells (arrowheads) within the marrow space. Signal and morphology are visualised using DAB as a substrate with a light haematoxylin counterstain. (*D*) Gelatinase B expression in an osteoclast adjacent to the bone surface (arrow) and in the marrow in what appears to be an osteoclast or a cluster of mononuclear cells (arrowheads). (*E–G*) Serial sections showing plump osteoblasts on trabeculae immunolocalised using an indirect peroxidase system with positive cells identified by the red colour reaction of AEC. (*E*) Cell-associated collagenase expression in osteoblasts (arrows) and in some osteocytes in the osteoid (arrowheads). (*F*) Matrix-associated stromelysin expression detected in the nonmineralised osteoid (asterisks), with little or no expression in the osteoblasts (arrows). (*G*) Very low level cell-associated TIMP staining in the osteoblasts. b, bone; o, osteoid; h, hypertrophic chondrocytes. Bars, 20 μm.

Table 1. *Summary of the distribution of MMPs and TIMP**

	CL	GL-A	GL-B	SL	TIMP
Fibrous tissue					
Mesenchymal cells	++	−	−	−	+
Fibroblasts	−	+	−	+++(m)	++
Vascular cells	++	−	−	+(m)	−/+
Cartilage/endochondral					
Proliferating chondrocytes	++	−	−	++(m)	++
Mature chondrocytes	+	−	−	+(m)	+
Hypertrophic chondrocytes	+	−	−	+	+
Vascular cells	++	−	−	−	+
Mineralised bone	+	−	+++(m)	+	++
Osteoblasts	+++	−	−	+	+
Intramembranous					
Fibroblasts	−	−	−	++(m)	+
Osteoblasts	+++	−	−	+(m)	++
Modelling/remodelling					
Osteoclasts	+	−	+++	−	−
Osteoblasts	+++	−	−	+	++
Osteocytes	+	−	−	+	−
Mononuclear cells	+++	−	+++	−	+
Lining cells	++	−	−	−	−

* All the osteophytes examined were similar in morphology and composed of 3 main types of tissue and cells: (1) fibrous-tissue with mesenchymal and vascular cells; (2) cartilage with chondrocytes and vascular cells; (3) endochondral and intramembranous bone formation and associated bone cells. −, no staining; +, less than 20% cells staining; ++, 20–50% cells staining; +++, more than 50% cells staining; m, mainly matrix staining; CL, collagenase; GL-A, gelatinase A; GL-B, gelatinase B; SL, stromelysin; TIMP, tissue inhibitor of matrix metalloproteinases.

Within the developing 'miniosteophyte' TRAP was observed in mononuclear cells; the central area exhibited large numbers of TRAP-positive multinucleated osteoclasts and there was intense staining of chondrocytes and osteocytes in the cartilage and bone. Strong staining for alkaline phosphatase was also evident in the plump osteoblasts at bone forming areas.

Immunolocalisation

Serial sections were assessed for collagenase, gelatinase A and B, stromelysin and TIMP-1 expression. Normal sheep serum (NSS) was used as a control. This sequence was repeated at 125 μm intervals throughout the osteophyte. The pattern of distribution for each antigen is described below and summarised in the Table. Control sections treated with NSS or absence of primary or secondary antibody showed absent or negligible staining (Fig. 2*A*, *B*).

Collagenase

Staining for collagenase was widespread, specific and intense in some areas (Table). Elongated flattened fibroblast-like cells in the mesenchymal tissue showed positive collagenase staining. In cartilage and fibrous tissue, distinct cell-associated collagenase expression was seen at sites of vascular invasion (Fig. 2*C*); osteoblasts in areas of endochondral and intramembranous bone formation showed strong staining whilst some, but not all, osteoclasts in these areas were positive. Osteoblasts laying down primary woven bone on calcified cartilage remnants demonstrated strong collagenase expression, as did the plump osteoblasts associated with thick osteoid seams (Fig. 2*E*). Chondrocytes in the proliferative and mature zones stained positively in some areas but were negative in others (Fig. 3*A*). Some highly vacuolated chondrocytes close to the mineralising bone, which were strongly positive for TRAP and alkaline phosphatase, also showed collagenase positivity (Fig. 3*B*). Occasional matrix-bound staining was observed in osteoid seams. Lining cells on some quiescent bone surfaces also exhibited positivity. Some osteoclasts in areas of bone remodelling expressed collagenase whilst others were negative.

Gelatinase A and B

Expression of gelatinase A was rarely observed. Occasional intense matrix-associated staining in fibro-

cartilaginous tissue surrounding areas of endochondral ossification was seen. In contrast gelatinase B showed intense, focal cell and matrix-associated expression in areas of active bone modelling and remodelling (Table). Many osteoclasts stained strongly (Fig. 2*D*), as did mononuclear cells in the marrow spaces and at sites of resorption. No staining was seen in the fibrocartilage, chondrocytes, osteoblasts or osteocytes (Table, Fig. 3*C*, *D*). In the miniosteophyte, examination of serial sections revealed that areas staining for stromelysin were gelatinase B negative; as the stromelysin signal decreased there was an increase in gelatinase B expression.

Stromelysin

Stromelysin was occasionally observed in some plump osteoblasts adjacent to the trabecular surface but mainly in the underlying osteoid (Fig. 2*F*). Matrix-associated staining for stromelysin was also detected in fibrous tissue. In cartilage adjacent to bone formation there was intense matrix-associated staining and cell associated expression in chondrocytes in the proliferative and mature zones (Fig. 3*E*). There was also staining for stromelysin in some, but not all, hypertrophic chondrocytes and osteocytes whilst the surrounding mineralised matrix exhibited only weak staining (Fig. 3*F*). From examination of serial sections it appeared that the expression of cell-associated stromelysin was transient but the matrix staining more permanent. Strong matrix staining was observed in the immature fibrous tissue of the developing 'miniosteophyte'.

TIMP-1

The expression of TIMP was widespread but variable in intensity. Staining was largely cell-associated, but some matrix staining was seen in osteoid. In areas of newly forming osteoid, strong collagenase and stromelysin expression was associated with only low levels of TIMP expression (Fig. 2*E*, *F*, *G*). The percentage of cells staining positively varied widely amongst chondrocytes at all stages of differentiation, the highest percentage being seen in the proliferating region of cartilage with a decrease towards the mineralising zone (Fig. 3*G*, *H*). At some sites of bone resorption there was intense matrix-associated signal and within the marrow spaces some of the mononuclear cells showed positive expression. Osteoblasts in areas of endochondral and intramembranous bone formation exhibited varying levels of staining, the lowest levels of expression being associated with areas of rapid modelling. No staining for TIMP was observed in osteoclasts.

DISCUSSION

Our results demonstrate that the MMPs and TIMP are widely and differentially distributed throughout developing human osteophyte tissue and indicate that they play specific roles in bone development. The growth of osteophytes is characterised by endochondral ossification, vascular invasion, cellular proliferation, differentiation and elaboration of intercellular matrix (Resnick & Niwayama, 1988; Dodds & Gowen, 1994), followed by advancing mineralisation and new bone formation. These changes resemble those seen in the epiphyseal growth plate of immature growing long bones, thus making osteophytes a useful model for studies of bone development (Merry et al. 1993; Aigner et al. 1995; Dodds et al. 1995; Middleton et al. 1995).

The demonstration of collagenase expression in osteoblasts and osteoclasts in osteophytic tissue is consistent with our previously reported results, on the basis of which we postulated multiple roles for collagenase in bone remodelling (Bord et al. 1996). The role of collagenase in bone resorption is uncertain; MMPs are optimally active at a neutral pH and are therefore unlikely to play a role in the initial stages of resorption, in which bone mineral is dissolved in a highly acid environment. However, later in the resorptive process the subcellular zone approaches a neutral pH as a result of the buffering action of bone salts and at this stage MMPs may be involved in matrix degradation. The observation, in the present study, that both collagenase and gelatinase B were expressed by osteoclasts, indicates that both enzymes may play a role in bone resorption and are consistent with their reported presence in osteoclasts from several species (Hill et al. 1994; Wucherpfennig et al. 1994; Rao et al. 1995; Witty et al. 1996).

In the present study, collagenase expression was observed in some chondrocytes and in osteoblasts close to areas of endochondral and intramembranous ossification, indicating possible roles in these processes and also in cartilage formation. The presence of collagenase at sites of vascular invasion is of particular interest in view of the well documented relationship between angiogenesis and osteogenesis; degradation of the basement membrane by collagenase may facilitate the migration of bone resorbing cells to sites of osteogenesis. Interestingly, stromelysin expression was also observed adjacent to sites of vascular

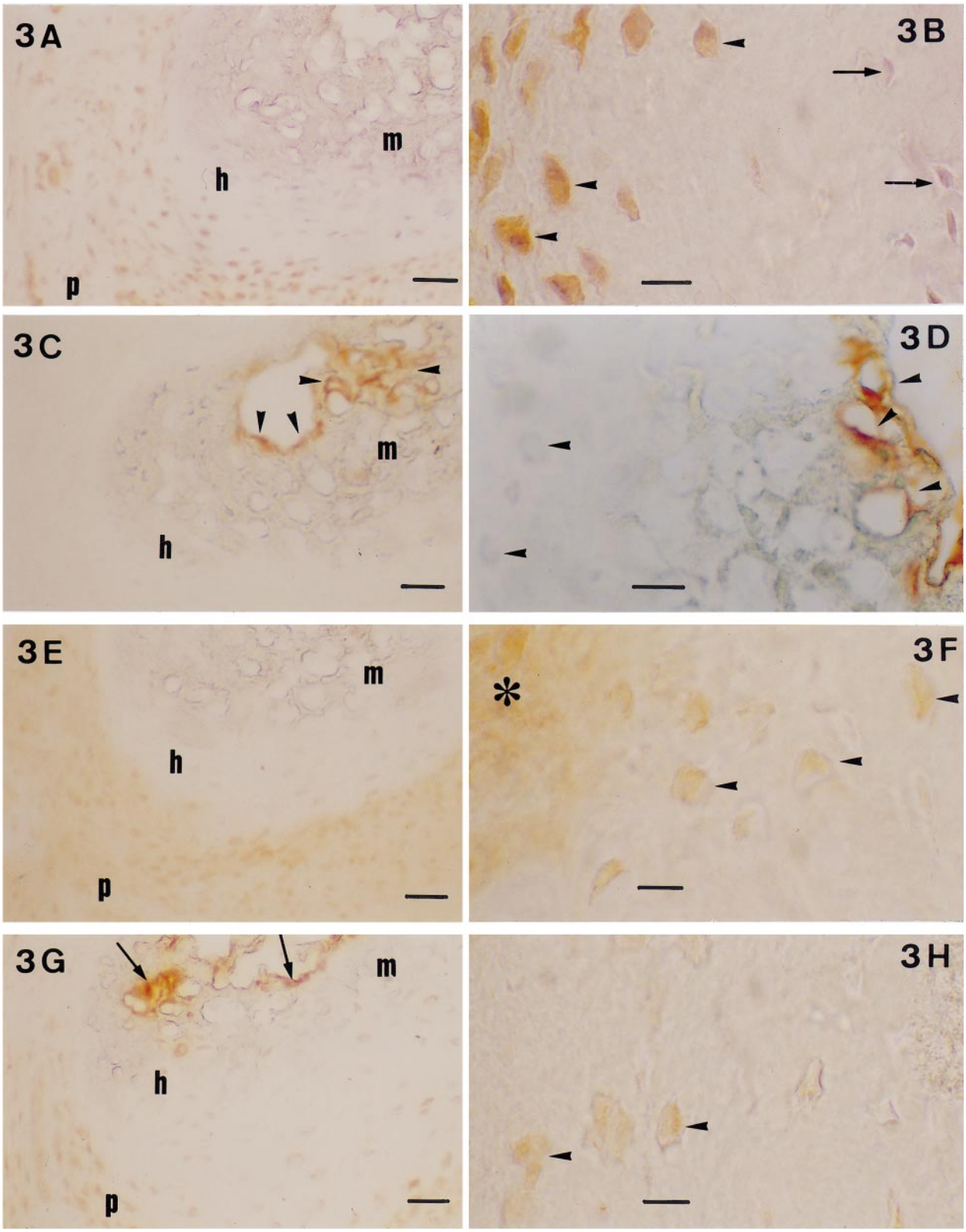

Fig. 3. Serial sections of an osteophyte showing endochondral ossification immunolocalised for MMPs and TIMP. Positive areas are visualised by the brown colour reaction of DAB at sites of antigen binding. (*A*) Cell-associated collagenase expression in chondrocytes surrounding the mineralising area. Strongest signal was observed in the proliferative and mature zones. (*B*) Higher power view of the mineralising area shows positive collagenase expression in some vacuolated chondrocytes (arrowheads) and occasional osteocytes (arrows). (*C*) Gelatinase B detected only in areas of mineralised bone (arrowheads). (*D*) Higher power view of (*C*) showing absence of staining in the chondrocytes (arrowheads). (*E*) Stromelysin expression in the matrix and cells in the proliferative chondrocytes. (*F*) High power view of (*E*) showing the mineralising and adjacent area, with low expression in some hypertrophic chondrocytes (arrowheads) and stronger expression in the matrix of the proliferative zone (asterisk). (*G*, *H*) Low and high power magnification showing strong TIMP immunolocalisation in the bony area (arrows) and weak cell-associated staining in some cells in the proliferative and mature chondrocytes (arrowheads). m, mineral; h, hypertrophic chondrocytes; c, mature chondrocytes; p, proliferating chondrocytes. Bars, *A*, *C*, *E*, *G*, 50 μm; *B*, *D*, *F*, *H*, 20 μm.

invasion, thus providing a means by which collagenase could be activated. Although this finding of collagenase in blood vessels is, to the best of our knowledge, the first report cited in human bone in vivo, it has been previously observed in blood vessel development in human fetal skin (Karelina et al. 1995), in healing human burn wounds (Stricklin & Nanney, 1994) and in advanced periodontitis (Pinchback et al. 1996).

Stromelysin binds to collagen both in its latent and active form, whilst TIMP-1 binds to the matrix-bound active enzyme but not to matrix-bound prostromelysin (Allan et al. 1991). The colocalisation of TIMP and stromelysin thus indicates that at least some of the enzyme present is active and the widespread distribution of stromelysin in matrix and fibrous tissue may reflect its role as an activator of collagenase (Murphy et al. 1987). Stromelysin was also expressed by osteoblasts in areas of bone formation, possibly indicating a more specific role in this process.

In contrast to our previous studies (Meikle et al. 1992, 1995) in which we demonstrated the constitutive production of gelatinase A by human osteoblasts in vitro, only very small amounts of this enzyme were detected in situ in this model, expression being confined to the fibrocartilaginous tissue surrounding the osteophyte. Breckon et al. (1995) reported extensive gelatinase A distribution in osteogenic tissue localised in the craniofacial region of the rabbit embryo. Whether these differences are due to species variation or their culture of explants and subsequent monensin treatment remains undecided. However, their observations with regard to gelatinase B are in agreement with our findings.

The differential staining patterns throughout the cartilage (summarised in the Table) suggest several functional roles. The high expression of stromelysin and collagenase in the proliferating chondrocytes could aid local degradation of the extracellular matrix, thus providing space for the increasing cell population. Proteoglycan is reported to inhibit calcification (DeBarnard et al. 1977; Blumenthal et al. 1979; Brown et al. 1989) and the presence of stromelysin in the hypertrophic chondrocytes suggests that the degradation of proteoglycans prior to mineralisation may be modulated by MMPs. During chondrocyte hypertrophy there is an increase in cell volume and a decrease in collagen content (Dean et al. 1989) and thus the presence of collagenase in these cells indicates that it may play a role in this course of events.

The widespread expression of TIMP-1 throughout bone and cartilage is likely to reflect its role as a regulator of metalloproteinase activity; the observation of an inverse relationship between collagenase and stromelysin expression on the one hand and TIMP staining on the other would be consistent with this hypothesis. Many other factors influence metalloproteinase activity including cytokines, systemic hormones, growth factors and plasminogen activators (Birkedal-Hansen et al. 1993). Further work is required to establish how these different factors interact with MMPs in the process of bone development.

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REFERENCES

Aigner T, Dietz U, Stob H, von der Mark K (1995) Differential expression of collagen types I, II, III and X in human osteophytes. *Laboratory Investigation* **73**, 236–243.

Allan JA, Hembry RM, Angal S, Reynolds JJ, Murphy G (1991) Binding of latent and high M_r active forms of stromelysin to collagen is mediated by the C-terminal domain. *Journal of Cell Science* **99**, 789–795.

Birkedal-Hansen H, Moore WGI, Bodden MK, Windsor LJ, Birkedal-Hansen B, Decarlo A et al. (1993) Matrix metalloproteinase: a review. *Critical Reviews in Oral Biological Medicine* **4** (2), 197–220.

Blumenthal NC, Posner AS, Silverman LD, Rosenberg LC (1979) Effect on proteoglycan on in vitro hydroxyapatite formation. *Calcified Tissue International* **27**, 75–82.

Bord S, Horner A, Hembry RM, Reynolds JJ, Compston JE (1996) Production of collagenase by human osteoblasts and osteoclasts in vivo. *Bone* **19**, 35–40.

Bradbeer J, Lindsay PC, Reeve J (1994) Fluctuation of mineral apposition rate at individual bone remodelling sites in human iliac cancellous bone: independent correlations with ostoid width and osteoblastic alkaline phosphatase activity. *Journal of Bone and Mineral Research* **9**, 1679–1686.

Breckon JJW, Hembry RM, Reynolds JJ, Meikle MC (1995) Matrix metalloproteinases and TIMP-1 localization at sites of osteogenesis in the craniofacial region of the rabbit embryo. *Anatomical Record* **242**, 177–187.

Brown CC, Hembry RM, Reynolds JJ (1989) Immunolocalisation of metalloproteinases and their inhibitor in the rabbit growth plate. *Journal of Bone and Joint Surgery* **71A**, 580–593.

Cawston TE (1986) Protein inhibitors of metalloproteinases. In *Proteinase Inhibitors* (ed. Barrett AJ, Salvesen G), pp. 589–610. Amsterdam: Elsevier.

Dean DD, Muniz OE, Howell DS (1989) Association of collagenase and tissue inhibitor of metalloproteinases (TIMP) with hypertrophic cell enlargement in the growth plate. *Matrix* **9**, 366–375.

DeBarnard B, Stagni N, Colautti I, Vittur F, Bonucci E (1977) Glycosaminoglycans and endochondral calcification. *Clinical Orthopaedics and Related Research* **126**, 285–291.

Delaissé JM, Eeckhout Y, Vaes G (1988) Bone resorbing agents affect the production and distribution of procollagenase as well as the activity of collagenase in bone tissue. *Endocrinology* **123**, 264–276.

Delaissé JM, Eeckhout Y, Neff L, François-Gillet CH, Henriet P, Su Y et al. (1993) (Pro)collagenase (matrix metalloproteinase-1) is present in rodent osteoclasts and in the underlying bone-resorbing compartment. *Journal of Cell Science* **106**, 1071–1082.

Dodds RA, Gowen M (1994) The growing osteophyte: a model system for the study of human bone development and remodelling in situ. *Journal of Histotechnology* **17**, 37–45.

Dodds RA, Connor JR, James IE, Rykaczewski E, Appelbaum E, Dul E et al. (1995) Human osteoclasts, not osteoblasts, deposit osteopontin onto resorption surfaces: an in vitro and ex vivo study of remodeling bone. *Journal of Bone and Mineral Research* **10**, 1666–1680.

Everts V, Delaissé JM, Korper W, Neohof A, Vaes G, Beertsen W (1992) Degradation of collagen in the bone resorbing compartment underlying the osteoclast involves both cysteine-proteinase and matrix metalloproteinases. *Journal of Cell Physiology* **150**, 221–231.

Heath JK, Atkinson SJ, Meikle MC, Reynolds JJ (1984) Mouse osteoblasts synthesize collagenase in response to bone-resorbing agents. *Biochimica et Biophysica Acta* **802**, 151–154.

Hembry RM, Murphy G, Reynolds JJ (1985) Immunolocalisation of tissue inhibitor of metalloproteinase (TIMP) in human cells. Characterisation and use of a specific antiserum. *Journal of Cell Science* **73**, 105–119.

Hembry RM, Bagga MR, Reynolds JJ, Hamblen DL (1995) Immunolocalisation studies on six matrix metalloproteinases and their inhibitors, TIMP-1 and TIMP-2, in synovia from patients with osteo- and rheumatoid arthritis. *Annals of Rheumatic Diseases* **54**, 25–32.

Hill PA, Murphy G, Docherty AJP, Hembry RM, Millican TA, Reynolds JJ (1994) The effects of selective inhibitors of matrix metalloproteinases (MMPs) on bone resorption and the identification of MMPs and TIMP-1 in isolated osteoclasts. *Journal of Cell Science* **107**, 3055–3064.

Hipps DS, Hembry RM, Docherty AJP, Reynolds JJ, Murphy G (1991) Purification and characterisation of human 72-kDa gelatinase (type IV) collagenase. Use of immunolocalisation to demonstrate the non-coordinated regulation of the 72 kDa and 95 kDa gelatinases by human fibroblasts. *Biological Chemistry, Hoppe-Seyler*, **372**, 287–296.

Karelina TV, Goldberg GI, Eisen AZ (1995) Matrix metalloproteinases in blood vessel development in human fetal skin and in cutaneous tumors. *Journal of Investigative Dermatology* **105**, 411–417.

Loveridge N, Dean V, Goltzman D, Hendy GN (1991) Bioactivity of parathyroid hormone and parathyroid hormone-like peptide: agonist and antagonist activities of amino-terminal fragments as assessed by the cytochemical bioassay and in situ biochemistry. *Endocrinology* **128**, 1938–1946.

Meikle MC, McGarrity AM, Thomson BM, Reynolds JJ (1991) Bone-derived growth factors modulate collagenase and TIMP (tissue inhibitor of metalloproteinases) activity and type 1 collagen degradation by mouse calvarial osteoblasts. *Bone and Mineral* **12**, 41–55.

Meikle MC, Bord S, Hembry RM, Compston JE, Croucher PI, Reynolds JJ (1992) Human osteoblasts in culture synthesise collagenase and other matrix metalloproteinases in response to osteotropic hormones and cytokines. *Journal of Cell Science* **103**, 1093–1099.

Meikle MC, Bord S, Hembry RM, Reynolds JJ (1995) The synthesis of collagenase, gelatinase-A (72 kDa) and gelatinase-B (95 kDa), and TIMP-1 and TIMP-2 by human osteoblasts from normal and arthritic bone. *Bone* **17**, 255–260.

Merry K, Dodds R, Littlewood A, Gowen M (1993) Expression of osteopontin mRNA by osteoclasts and osteoblasts in modelling human bone. *Journal of Cell Science* **104**, 1013–1020.

Middleton J, Arnott N, Walsh S, Beresford J (1995) Osteoblasts and osteoclasts in adult human osteophyte tissue express the mRNAs for insulin-like growth factors I and II and the type I IGF receptor. *Bone* **16**, 287–293.

Murphy G, Cockett MI, Stephens PE, Smith BJ, Docherty AJP (1987) Stromelysin is an activator of procollagenase. A study with natural and recombinant enzymes. *Biochemical Journal* **248**, 265–268.

Murphy G, Ward R, Hembry RM, Reynolds JJ, Kuhn K, Tryggvason K (1989) Characterization of gelatinase from pig polymorphonuclear leucocytes. A metalloproteinase resembling tumour type IV collagenase. *Biochemical Journal* **258**, 463–472.

Murphy G, Allan JA, Willenbrock F, Cockett MI, O'Connell JP, Docherty AJP (1992) The role of the C-terminal domain in collagenase and stromelysin specificity. *Journal of Biological Chemistry* **267**, 9612–9618.

Murphy G, Reynolds JJ (1993) Extracellular matrix degradation. In *Connective Tissue and Its Heritable Disorders* (ed. Royce P, Steinmann B) pp. 287–316. New York: Wiley-Liss.

Pinchback JS, Gibbins JR, Hunter N (1996) Vascular co-localisation of proteolytic enzymes and proteinase inhibitors in advanced periodontitis. *Journal of Pathology* **179**, 326–332.

Raina V (1972) Normal osteoid tissue. *Journal of Clinical Pathology* **25**, 229–232.

Rao VH, Bridge JA, Neff JR, Schaefer GB, Buehler BA, Vishwanatha JK et al. (1995) Expression of 72 kDa and 92 kDa type IV collagenases from human giant-cell tumour of bone. *Clinical and Experimental Metastasis* **13**, 420–426.

Resnick D, Niwayama G (1995) Degenerative disease of extraspinal locations. In *Diagnosis of Bone and Joint Disorders*, 3rd edn, vol. 3 (ed. Resnick D), pp 1263–1372. Philadelphia: WB Saunders.

Stricklin GP, Nanney LB (1994) Immunolocalization of collagenase and TIMP in healing human burn wounds. *Journal of Investigative Dermatology* **103**, 488–492.

Whitham SE, Murphy G, Angel P, Rahmsdorf HJ, Smith BJ, Lyons A et al. (1986) Comparison of human stromelysin and collagenase by cloning and sequence analysis. *Biochemical Journal* **240**, 913–916.

Witty JP, Foster SA, Stricklin GP, Matisian LM, Stern PA (1996) Parathyroid hormone-induced resorption in fetal rat limb bones is associated with production of the metalloproteinases collagenase and gelatinase-B. *Journal of Bone and Mineral Research* **11**, 72–78.

Wucherpfennig AL, Li Y-P, Stetler-Stevenson WG, Rosenburg AE, Stashenko P (1994) Expression of 92 kD type IV collagenase/gelatinase B in human osteoclasts. *Journal of Bone and Mineral Research* **9**, 549–556.