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Expression of bone morphogenetic proteins and cartilage-derived morphogenetic proteins during osteophyte formation in humans

Sanja Zoricic,[1] Ivana Maric,[1] Dragica Bobinac[1] and Slobodan Vukicevic[2]

[1]*Department of Anatomy, School of Medicine, University of Rijeka, Croatia*
[2]*Department of Anatomy, School of Medicine, University of Zagreb, Croatia*

Abstract

Bone- and cartilage-derived morphogenetic proteins (BMPs and CDMPs), which are TGFβ superfamily members, are growth and differentiation factors that have been recently isolated, cloned and biologically characterized. They are important regulators of key events in the processes of bone formation during embryogenesis, postnatal growth, remodelling and regeneration of the skeleton. In the present study, we used immunohistochemical methods to investigate the distribution of BMP-2, -3, -5, -6, -7 and CDMP-1, -2, -3 in human osteophytes (abnormal bony outgrowths) isolated from osteoarthritic hip and knee joints from patients undergoing total joint replacement surgery. All osteophytes consisted of three different areas of active bone formation: (1) endochondral bone formation within cartilage residues; (2) intramembranous bone formation within the fibrous tissue cover and (3) bone formation within bone marrow spaces. The immunohistochemistry of certain BMPs and CDMPs in each of these three different bone formation sites was determined. The results indicate that each BMP has a distinct pattern of distribution. Immunoreactivity for BMP-2 was observed in fibrous tissue matrix as well as in osteoblasts; BMP-3 was mainly present in osteoblasts; BMP-6 was restricted to young osteocytes and bone matrix; BMP-7 was observed in hypertrophic chondrocytes, osteoblasts and young osteocytes of both endochondral and intramembranous bone formation sites. CDMP-1, -2 and -3 were strongly expressed in all cartilage cells. Surprisingly, BMP-3 and -6 were found in osteoclasts at the sites of bone resorption. Since a similar distribution pattern of bone morphogenetic proteins was observed during embryonal bone development, it is suggested that osteophyte formation is regulated by the same molecular mechanism as normal bone during embryogenesis.

Key words BMPs; CDMPs; human osteophyte; immunohistochemistry.

Introduction

Osteophytes are abnormal bony outgrowths developed mostly on the margin of articular surfaces during progression of osteoarthritis (OA) of large joints (Jeffery, 1975; Resnick, 1983). It is widely accepted that they represent repair attempts, and therefore could serve to protect affected joints against the progression of destruction (van der Berg et al. 1993). Although they are pathological features, it has been proposed that their development follows a basic pattern of both endochondral and intramembranous bone formation reminiscent of embryonal bone development, with an abnormal stimulus for growth (Dodds & Gowen, 1994a; Aigner et al. 1995). Since several studies have demonstrated that an osteophyte contains all the components of the bone remodelling cycle, it was suggested that osteophyte growth should be adopted as a human *in-situ* model for studying bone cell differentiation and function during development and remodelling (Dodds & Gowen, 1994a). However, little is known about the factors and molecular mechanisms inducing, promoting and regulating osteophyte growth itself. Growth factors detected during osteophyte formation include insulin-like growth factors (IGFs) (Middleton et al. 1995),

Correspondence
Dr Sanja Zoricic, MD, PhD, Department of Anatomy, School of Medicine, University of Rijeka, B. Branchetta 20, 51000 Rijeka, Croatia. Tel. + 385 51 651143; Fax: + 385 51 651143; E-mail: sanja@mamed.medri.hr

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transforming growth factor type β1 (TGF β1) (Dodds et al. 1994b), type β2 and β3 (TGF β2 and TGF β3) (Horner et al. 1998), platelet-derived growth factor (PDGF) (Horner et al. 1996) and interleukines IL-1β and IL-6 (Dodds et al. 1994b). It has been observed that TGF β1 injected into murine knee joint induces osteophyte formation (van Beuningen et al. 1994). In these studies, the expression of certain growth factors in osteophytes was used in an attempt to understand bone development and remodelling, rather than as a model for understanding the role of growth factors in the development and growth of osteophytes during the course of OA.

Bone morphogenetic proteins (BMPs) play an important role in bone growth and development (Wozney et al. 1988; Reddi, 1998; Wozney & Rosen, 1998). They have the ability to induce and promote new cartilage and bone formation at an ectopic site. *In vivo*, BMPs are expressed in the cells of developing bones (Vukicevic et al. 1994b; Helder et al. 1995), in the fracture callus (Nakase et al. 1994; Bostrom et al. 1995; Onishi et al. 1998) and in the ectopic bone formation induced by implanted recombinant BMPs (Sampath et al. 1993; Sampath & Reddi, 1981). It was also shown that BMP-2/4, -3, -5, -6 and -7 are important regulators of skeletal tissue formation and repair (Wozney et al. 1990; Cook et al. 1994; Riley et al. 1996; Cook, 1999; Aspenberg et al. 2000; Fujimoto et al. 2001). Cartilage-derived morphogenetic proteins (CDMP-1, -2 and -3; also known as BMP-14, -13, -12), a BMP subgroup, are essential for the formation of cartilaginous tissue during early limb development (Luyten, 1995; Chang et al. 1994) and for the formation of the articular joint cavity during joint development. They were also found to be expressed in adult normal and osteoarthritic articular cartilage which suggests their role in the maintenance and regeneration of the articular cartilage (Erlacher et al. 1998). Since BMP members have predominant osteoinductive properties, they may play a role in osteophyte formation in OA joints. In our study we explored the distribution of BMPs and CDMPs in human osteophytes at different stages of endochondral and intramembranous ossification.

Materials and methods

Tissue preparation

The study was performed on a series of 20 osteophytes removed from the femoral heads and tibial plateaus of individuals undergoing joint replacement surgery due to osteoarthritic lesions of knee and hip joints. The age of the patients ranged from 60 to 85 years. Informed written consent and approval was obtained from the local ethics committee. The osteophytes were recognized as small overhanging lips located at the edges of articular surfaces. They were dissected out with their underlying bone, and their basal sides were marked. After being isolated from the surrounding tissue, the osteophytes were washed in saline and immediately fixed in 4% paraformaldehyde for several hours. Two different protocols were used for further specimen processing. In the first, one half of each osteophyte was embedded (without going through decalcification) in a methyl methacrylate, and the whole sample was than serially sectioned at 200-μm intervals. Sectioning was performed on a rotatory microtome (Leica RM 2155) equipped with a tungsten carbide knife. Sections were than stained either for haematoxylin and eosin in order to examine general morphology, or with toluidine blue and safranine O to determine proteoglycan content. In the second protocol, the other half of each osteophyte was decalcified in 10% EDTA and embedded in paraffin wax. Tissue slices (3–4 μm) were collected on 3-aminopropyltriethoxy-silane (APES, Sigma)-coated glass slides, air-dried and stored at 4 °C until processing for immunohistochemistry.

Primary antibodies

Anti-BMP-2 and -5 were goat polyclonal antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Both anti-BMP-2 and -5 were raised against amino terminal regions of BMP-2 and -5 of human origin, and did not cross-react with other BMPs. Anti-BMP-3 was a rabbit polyclonal antibody (FS-3) raised against the N-terminus of mature BMP-3 monomer (amino acids 329–348), and was specific for BMP-3 (Vukicevic et al. 1994a). Anti BMP-7 was a monoclonal antibody (12G3) generated by hyperimmunizing mice with purified CHO-expressed disulphide-linked mature BMP-7 homodimers, and did not show cross-reactivity (Sampath et al. 1992). The anti-CDMP-1, -2 and -3 antibodies were a gift from TK Sampath (Creative BioMoleculas, Hopkinton, USA). Anti-CDMP-1 polyclonal antibody was generated in rabbits using the synthetic peptide of 13 amino acids of the N-terminus of the mature human CDMP-1 monomer; anti-CDMP-2 polyclonal antibody was raised in rabbits using specific peptide (amino acid

residue 386–399), and anti-CDMP-3 polyclonal antibody was produced in rabbits against the N-terminus of a CDMP-3 monomer. Anti-BMP-6 was a polyclonal antibody raised in rabbits against the C-terminus of BMP-6 (a gift from T. K. Sampath, Creative BioMoleculas).

Immunohistochemistry

Immunohistochemistry was carried out using an indirect immunoperoxidase system. Tissue slices were deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidase and non-specific binding were blocked by incubation in 0.3% H_2O_2 in methanol for 30 min followed by extensive washing in phosphate-buffered saline (PBS). In order to decrease background staining, the incubation in 5% non-immune serum (30 min at 4 °C in a humid chamber) was also performed prior to incubation in primary antibodies. After incubation with a primary antibody (60 min at room temperature) the tissue was washed in PBS and the secondary biotinylated antibody was applied according to the manufacturer's protocol (DAKO, LSAB® + Kit Peroxidase). Peroxidase conjugated streptavidin was added, and the site of antigen binding was visualized using 3-aminoethylcarbazole (AEC) as chromogen producing a purple red stain. Sections were counterstained with haematoxylin. Slides used as a control were processed either with normal serum replacing specific primary antibodies or just with the secondary antibody alone. Sections were mounted in Histostain and analysed with an Olympus BX 50 microscope (Olympus, Japan). The microphotographs were obtained using an Olympus OM-4 Ti camera.

Results

Morphology of the osteophytes

Osteophytes, collected from the margin of articular surfaces of the femoral head and tibial plateau, were different in size, shape and position. Regardless of their localization, they showed similar morphological features. According to the morphological criteria based on toluidin blue and safranine O matrix staining patterns, three types of connective tissues were found within osteophytes: fibrous, cartilage and bone tissue (Fig. 1a). Fibrous connective tissue, containing fibroblast-like cells surrounded by a toluidine blue and safranine-O-negative extracellular matrix with numerous blood vessels, was located usually at the periphery of an osteophyte, merging with adjacent synovial tissue. In some samples, the fibrous tissue extended over the entire surface of an osteophyte, covering underlying cartilaginous tissue located in the upper part of the osteophyte. Consequently, the basal side of the fibrous tissue displayed a transitional layer of fibrocartilage containing numerous round and spindle-shaped cells embedded in toluidine blue and safranin-O-positive extracellular matrix, which was irregularly structured. Cartilaginous tissue had a typical morphology of a hyaline cartilage. In the lower parts of the cartilage, hypertrophic chondrocytes were arranged in columns, similar to those found in the proliferative zone of a growth plate or in the articular cartilage. A bone comprised the newly formed woven bone in the superficial zones and the mature lamellar trabeculae in deeper areas. Both endochondral and intramembranous bone formation patterns were present in each sample. The basal side of the cartilage layer was invaded by vascular connective tissue from the underlying bone (Fig. 1b). On the surface of eroded cartilage, numerous osteoclasts and osteoblasts were observed. Newly formed woven bone trabeculae containing a cartilaginous core emanated directly from the eroded cartilage surface. Superficially, the fibrous connective tissue covering the underlying lamellar bone (Fig. 1c) contained round fibroblast-like cells, which gradually transformed into osteoblasts embedded in osteoid seams. The newly formed trabecular bone in the deeper parts contained highly cellular marrow spaces (Fig. 1d). Numerous osteoblasts were forming a layer of osteoid over bone trabeculae.

Endochondral bone

Certain bone- and cartilage-derived morphogenetic proteins were differentially expressed at the sites of endochondral bone development within an osteophyte (Table 1). BMP-7 localization was specific and restricted to hypertrophic chondrocytes (Fig. 2a) and osteoblasts of the newly formed woven bone trabeculae (Fig. 2b). Occasionally, BMP-7 immunostaining was observed in chondrocytes of proliferating and mature layers and in the surrounding matrix. Discrete BMP-7 staining was occasionally found in young osteocytes embedded in woven bone, but osteocytes and lining cells of the lamellar bone were BMP-7 negative. BMP-3 was found at a similar localization as BMP-7 (Fig. 2c).

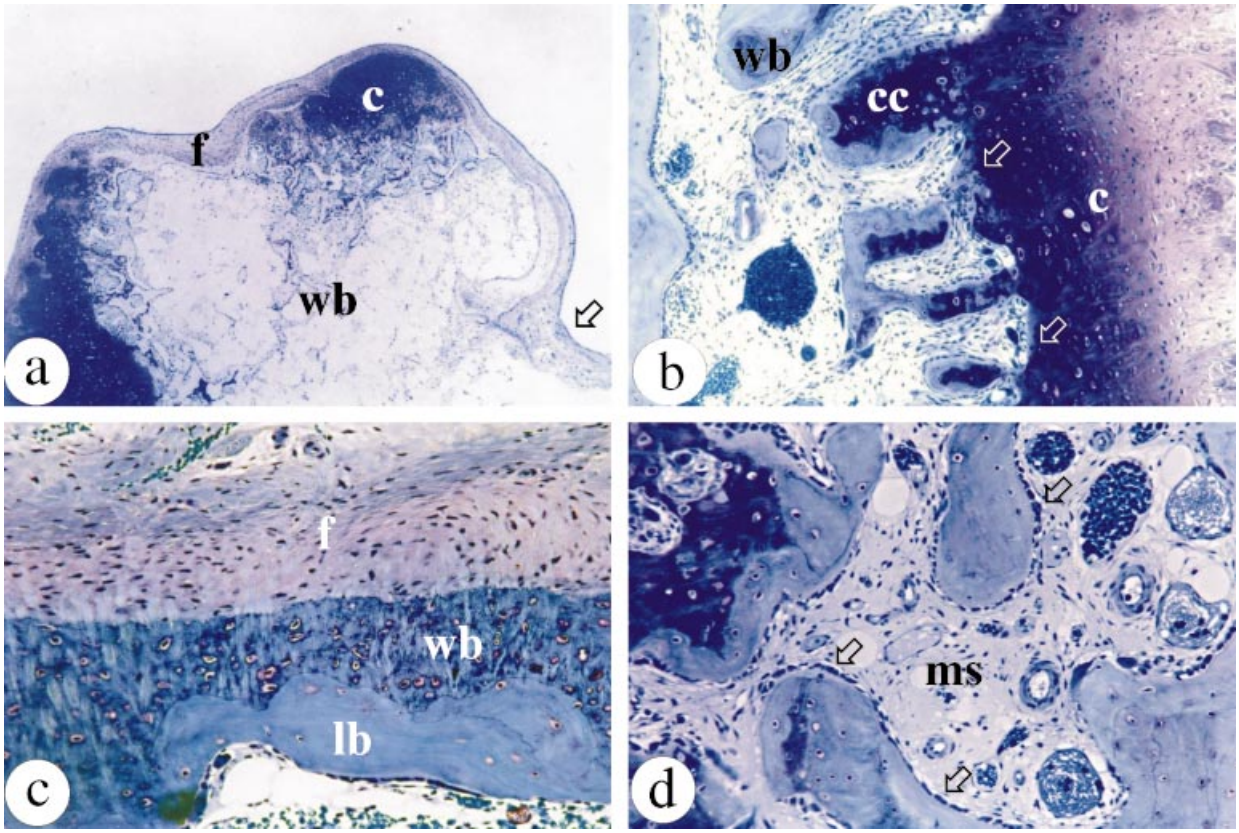

Fig. 1 (a) Cross-section of typical osteophyte from hip joint, showing three main types of tissue and typical arrangement of the zones as distinguished by histomorphological criteria and olivine blue staining pattern: fibrous tissue (f), cartilage (c), bone (wb). The peripheral rim of the osteophyte merging with the synovial tissue (arrow) is covered by fibrous tissue and cross-passing blood vessels, with this fibrous tissue also extending over the surface layer of the cartilaginous tissue (toluidine blue, magnification 40×). (b) On the eroded side (arrow) of the cartilage (c), numerous osteoclasts, osteoblasts and highly cellular marrow stromal tissues were present; the newly formed woven bone trabeculae (wb) still contain areas of residual cartilaginous core (cc), clearly indicating endochondral bone development (toluidine blue, magnification 200×). (c) Intramembranous bone formation on the surface of the osteophyte; this fragment shows clusters of mesenchymal cells within connective fibrous (f) tissue that have gradually become enlarged and embedded in unmineralized woven bone matrix (wb), laying down the pre-existing mineralized lamellar bone trabeculae (lb) (toluidine blue, magnification 200×). (d) Specific area of active remodelling within osteophytes showed that within marrow containing connective tissue, there is differentiation of marrow cells into osteoblasts (arrow). As a result of this cell differentiation, new bone trabeculae arise on the free surface of old bone trabeculae. These two 'types' of trabeculaes can be distinguished, as shown clearly on this fragment found within the osteophyte (toluidine blue, magnification 200×).

Unexpectedly, several osteoclasts with the surrounding matrix stained for BMP-3. Conversely, BMP-5 and BMP-6 were both detected mainly in osteoblasts and in young osteocytes (Fig. 2d,e). BMP-5 immunostaining was also found in several chondrocytes at the ossification front and BMP-6 was found in osteoclasts. BMP-2 was detected in the cells within the blood vessel wall invading the basal side of the cartilage, with chondrocytes, osteoblasts and matrix being negative. All three CDMPs have a similar pattern of distribution and were detected in the majority of chondrocytes and the cartilage matrix (Fig. 2f).

Intramembranous bone

At the osteophyte periphery, bone formation occurred by the process of intramembranous formation which showed a different pattern of morphogenetic protein distribution compared to endochondral bone formation (Table 1). Fibroblasts and the surrounding matrix showed the most intense staining for BMP-2 (Fig. 3a) and BMP-3 (Fig. 3b), and did not stain for BMP-5, -6, -7 and CDMPs. Osteoblasts and young osteocytes of the newly formed intramembranous bone stained for all tested BMPs and did not stain for the CDMPs.

Table 1 Expression of BMPs and CDMPs in developing human osteophytes from knee and hip joint

	BMP-2	BMP-3	BMP-5	BMP-6	BMP-7	CDMP-1	CDMP-2	CDMP-3
Endochondral bone formation								
Proliferating and mature chondrocytes	–	–	–	–	+ (m)	+++ (m)	+++ (m)	+++ (m)
Hypertrophic chondrocytes	–	+	–	–	+++	++	++	++
Osteoblasts	+	++	+++	++	+++	–	–	–
Newly formed bone osteocytes	–	+	+	+	+	–	–	–
Intramembranous bone formation								
Fibroblasts	+++ (m)	+ (m)	–	–	–	–	–	–
Osteoblasts	+	++	++	++	+++	–	–	–
Osteocytes	–	–	+	+	++	–	–	–
Modelling/remodelling sites								
Osteoblasts	–	+	++	+	++	–	–	–
Osteocytes	–	–	–	–	+	–	–	–
Osteoclasts	–	++	–	++ (m)	–	–	–	–
Quiescent bone surfaces								
Linning cells	–	–	–	–	–	–	–	–
Osteocytes	–	–	–	–	–	–	–	–

+++, maximal; ++, moderate; +, minimal; m, matrix staining; –, no staining.

Bone remodelling sites

Osteoblasts within remodelling sites of osteophytic bone stained positive for all tested BMPs, except for BMP-2 (Table 1). Osteocytes did not stain for BMPs and CDMPs except for BMP-7. Several osteoclasts stained positive for BMP-3 and BMP-6 (Fig. 3c,d).

Discussion

In this study we have presented evidence demonstrating that BMP-2, -3, -5, -6, -7, CDMP-1, -2 and -3 are expressed in osteophytic tissue obtained from human osteoarthritic joints. Furthermore, these growth factors exhibit distinct patterns of expression in the growing osteophyte. BMPs are predominantly localized in the cytoplasm, but occasionally they are also detected in the bone and cartilage extracellular matrix. BMPs are most frequently detected in osteoblasts, mature and hypertrophic chondrocytes, as well as perivascular cells. Therefore, our findings are consistent with data from other authors who found similar BMP expression during embryonic bone development and bone regenerative processes (Chang et al. 1994; Vukicevic et al. 1994a; Helder et al. 1995, 1998; Hogan, 1996; Reddi, 1998; Fujimoto et al. 2001). Our study confirmed some earlier work about osteophyte structures and morphological patterns of osteophyte growth and development (Aigner et al. 1995; Jeffery, 1975; Resnick, 1983; Dodds & Gowen, 1994). All osteophytes analysed in this study showed endochondral sequences of bone formation occurring within cartilage residues and/or newly formed cartilaginous tissue. There was also evidence of intramembranous bone formation within the covering fibrous tissue, and bone formation within the marrow spaces. Therefore, tissues involved in osteophyte growth and development go through the same morphogenetic processes as in embryonal life. Nevertheless, endochondral and intramembranous sites of bone formation in osteophytes were located near each other, indicating phenomena not generally observed elsewhere in the skeletal system.

It is well established that bone and cartilaginous cells produce BMPs. These morphogenetic proteins act as autocrine and/or paracrine factors regulating bone growth and remodelling (Centerella et al. 1994; Reddi, 1992, 1993; Manolagas & Jilka, 1995). BMPs promote and control proliferation and differentiation of the skeletal cells. BMP-2, -4, -6 and -7 induce ectopic bone formation in rats and mice if implanted subcutaneously (Wang et al. 1990; Sampath & Reddi, 1981). Different BMPs are expressed during embryonal skeletal development in various mammals (Hogan, 1996), and in bone cells during fracture repair and repair of the articular cartilage (Nakase et al. 1994; Bostrom et al. 1995; Urist et al. 1997). Recently, BMP-2 expression was demonstrated in fibroblastic cells and chondrocytes in the growth of a cartilage cap in osteochondroma (Nakase et al. 2001). Their findings suggest that BMP-2 locally induces or promotes chondrogenesis. Our results

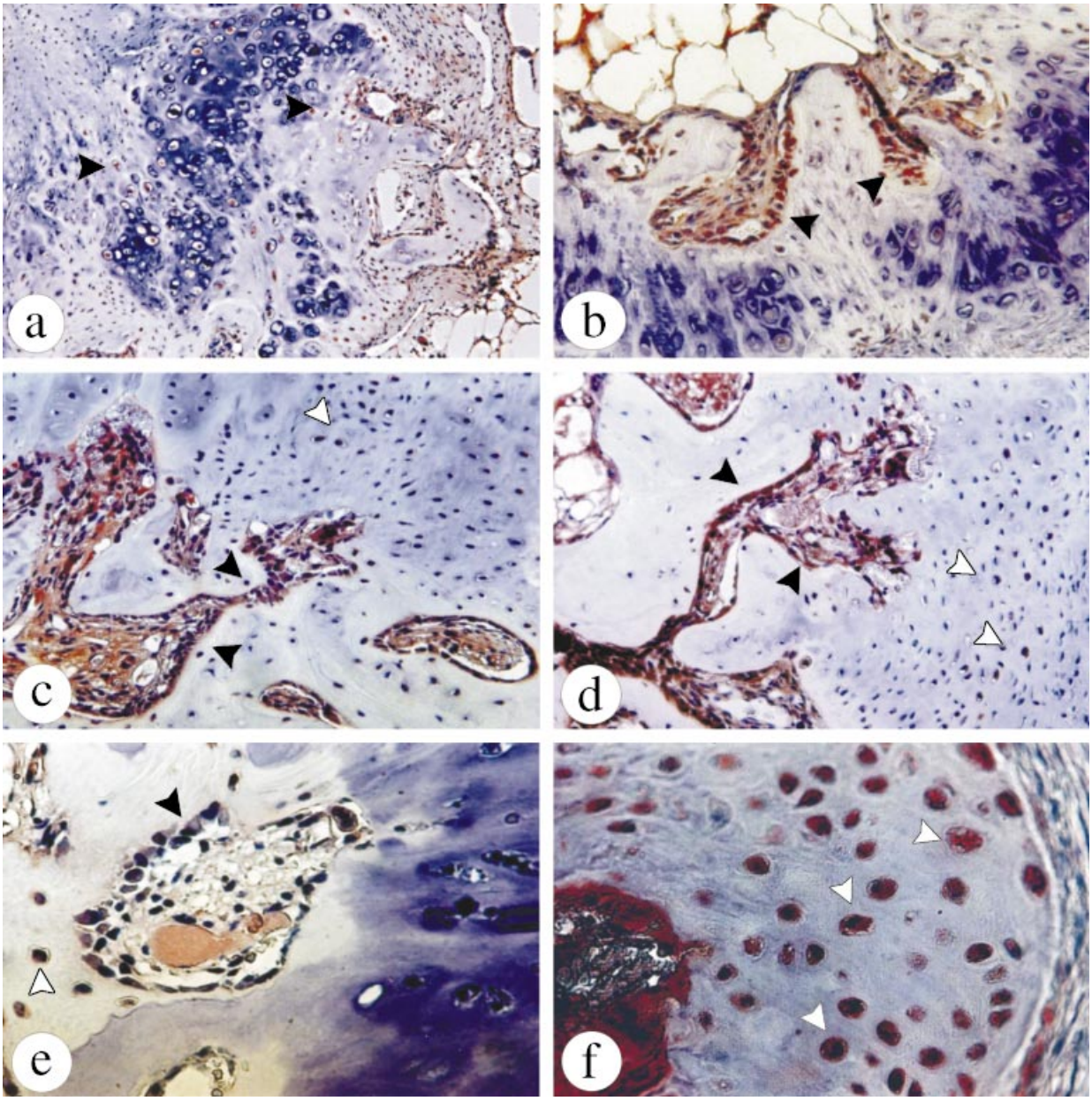

Fig. 2 BMPs immunostaining in the areas of endochondral bone development within osteophytes. (a) BMP-7 immunostaining was most intense in the cytoplasm of hypertrophic chondrocytes (arrowheads; magnification 100×). (b) Immunostaining in osteoblasts (arrowheads) of the newly formed woven bone trabeculae on the eroded side of the cartilage (magnification 200×). (c) BMP-3 immunostaining was found in some hypertrophic chondrocytes (white arrowheads), osteoblasts and young osteocytes (black arrowheads) (magnification 100×). (d) BMP-5 immunostaining was found in chondrocytes (white arrowheads) at the site of vascular invasion and matrix resorption, and in osteoblasts (black arrowheads) at the ossification front (magnification 100×). (e) BMP-6 immunostaining in osteoblasts (black arrowheads) and osteocytes (white arrowheads) of the newly formed woven bone trabeculae and in a minority of the chondrocytes (magnification 400×). (f) CDMP-1 immunostaining in osteophytes showed that all cartilaginous cells (black arrowheads) of the cartilage subjected to the endochondral bone development cascade were CDMP-1 positive; the covering fibrous tissue and underlying bone tissue showed no CDMP-1 immunostaining (magnification 400×).

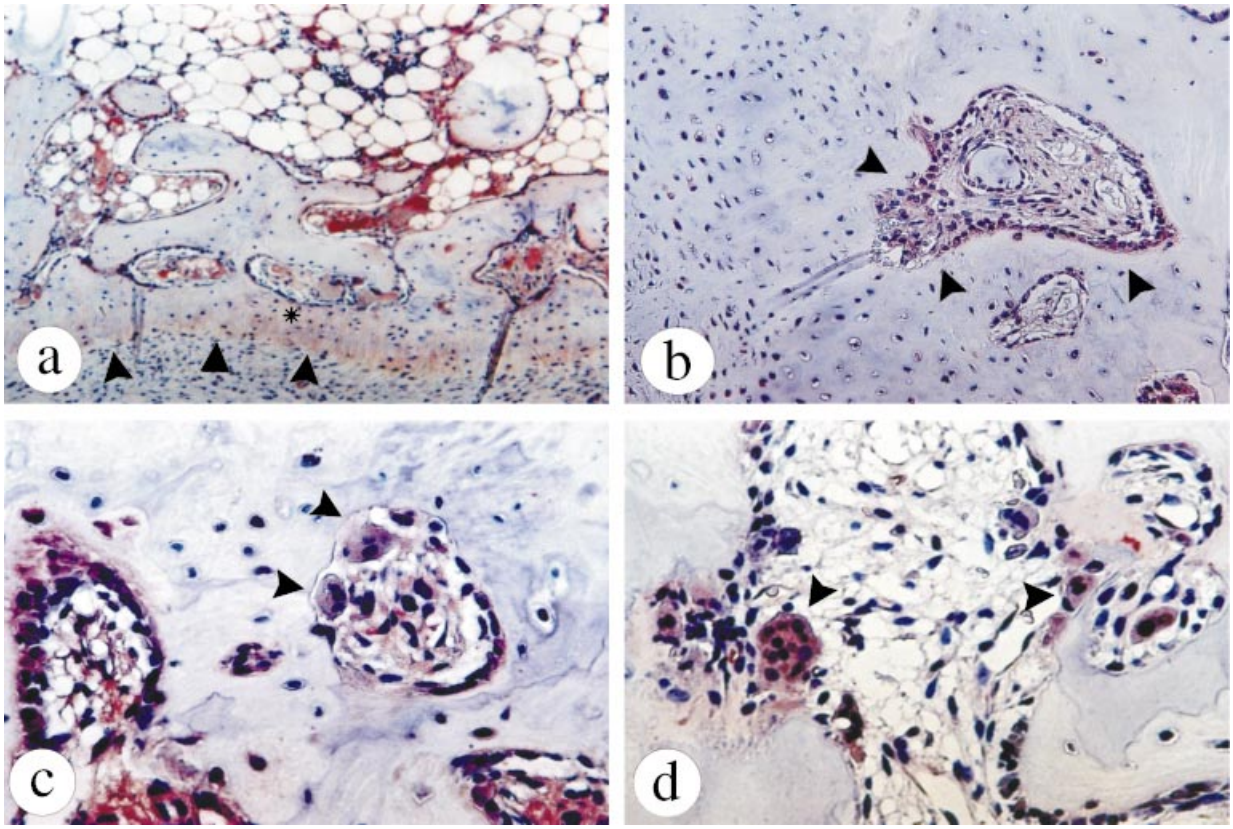

Fig. 3 (a) Matrix staining for BMP-2 was observed in the cells (arrowheads) of the superficial part of the covering fibrous tissue (asterisk) and in the matrix of the newly developed intramembranous bone (magnification 40×). (b) BMP-3-positive immunostaining in osteoblasts (arrowheads) at the sites of intramembranous bone formation (magnification 100×). BMP-3 positive (c, magnification 200×) and BMP-6 positive (d, magnification 400×) immunostaining were observed in osteoclasts (arrowheads) active in bone remodelling sites.

showed BMP-2 expression in hypertrophic chondrocytes at the sites of endochondral bone formation. This result supports another hypothesis that BMP-2 might regulate apoptosis during skeletal development (Anderson et al. 2000; Nakase et al. 2001).

Specific expression of certain BMPs in osteophytic tissues suggests osteoinductive signalling of BMPs within OA lesions. Our results have identified possible sites of production and action of these growth factors *in vivo*, providing an insight into their role in the growth and development of an osteophyte. Since these proteins have been normally expressed during embryonal skeletal development, their presence in osteophytic tissues may indicate that osteophytic growth is regulated by the same molecular mechanisms as during embryonal bone development. BMPs may have a specific role in the pathogenesis of osteoarthritis.

Recently, BMP expression was also demonstrated in bone-resorbing cells: osteoclasts. There is no precise explanation of their role in osteoclastic activity. Anderson et al. (1999, 2000) have demonstrated immunoreactivity for BMP-1–7 in osteoclasts involved in bone remodelling processes in human fetal and rat metaphysis. Furthermore, BMP-4 and -6 expression was shown in osteoclasts within the growth plate (Hodges et al. 1997; Anderson et al. 1999), with BMP-2 in osteoclasts present in the ectopic bone proliferations induced by periosteum (Nishimura et al. 1997), BMP-2, -4 and -7 in osteoclasts of regenerated bone during fracture repair (Onishi et al. 1998), and finally BMP-7 in osteoclasts of the alveolar bone (Helder et al. 1998). Our study demonstrates the presence of immunoreactivity for BMP-3 and BMP-6 in osteoclasts involved in remodelling processes during osteophyte formation. There are two possible explanations for the presence of BMPs in osteoclasts: either they are endogenously produced or they enter the osteoclasts during their phagocytic activity (Anderson et al. 2000). Whatever the origins of BMPs in osteoclasts, our immunohistochemical findings represent a new contribution to the body of evidence

suggesting that BMPs could be important factors in the regulation of osteoclastic activity. In the present study, BMP expression has also been found in bone matrix. This result is consistent with the hypothesis of Manolagas & Jilka (1995) that osteoclastic resorption of cartilage and bone matrix could release BMPs stored in extracellular matrix. This assumption might suggest the promotion of the osteophytic bone growth. Similarly, Anderson et al. (2000) suggested that BMPs released from resorbed matrix could be an important component of the intercellular signalling that leads to osteoblastic differentiation.

In conclusion, our results indicate that BMPs and CDMPs are involved in osteophyte formation during the pathogenesis of OA in humans. Even though osteophytes are pathological features, the observation that BMP expression is similar to that of embryonal and regenerative bone suggests that osteophytes represent attempted tissue repair within the OA joint.

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