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Localization of bone morphogenetic protein-2 in human osteoarthritic cartilage and osteophyte

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Summary

Objectives: To examine the localization of bone morphogenetic protein (BMP)-2 mRNA and protein in human osteoarthritic (OA) articular cartilage and osteophyte.

Design: Five normal, four growing and 14 OA human cartilage samples, graded histomorphologically by Mankin Score, were studied by *in situ* hybridization and immunohistochemistry for the expression of BMP-2.

Results: BMP-2 mRNA was present in chondrocytes in neonatal growing articular cartilage, but was scarcely present in normal adult articular cartilage. In OA articular cartilage, BMP-2 mRNA and protein were detected in both clustering and individual chondrocytes in moderately or severely damaged OA cartilage. In moderately damaged OA cartilage, BMP-2 mRNA was localized in both upper and middle zone chondrocytes, but was not detected in deep layer chondrocytes. In severely damaged OA cartilage, cellular localization of BMP-2 mRNA was extended to the deep zone. In the area of osteophyte formation, BMP-2 mRNA was intensely localized in fibroblastic mesenchymal cells, fibrochondrocytes, chondrocytes and osteoblasts in newly formed osteophytic tissue. The pattern of BMP-2/4 immunolocalization was associated with that of mRNA localization.

Conclusions: BMP-2 mRNA and BMP-2/4 were detected in cells appearing in OA tissues. BMP-2 was localized in cells of degenerating cartilage as well as osteophytic tissue. Given the negative localization of BMP-2 in normal adult articular cartilage, BMP-2 might be involved in the regenerating and anabolic activities of OA cells, which respond to cartilage damage occurring in osteoarthritis.

Key words: Osteoarthritis, Bone morphogenetic protein, Osteophyte.

Introduction

Osteoarthritic (OA) change in cartilage is characterized by loss of matrix molecules including proteoglycans and collagen[1,2]. However, the cells that appear during these degenerative events are thought to have anabolic actions, such as proliferation and synthesis of cartilage matrix components[1–3]. These cells also reportedly regain the chondrogenic phenotype and are committed to the regenerative pathway, based on the results that Col IIA, a marker for chondroprogenitor cells, has been reexpressed in OA cells[4].

Various factors may potentiate and regulate this regenerating action of OA cells. One of the potent chondrogenic factors, transforming growth factor-β (TGF-β), was reported to play a role in the process of osteophyte formation in animal models[5,6] and is expressed in human OA cartilage[7]. Bone morphogenetic protein (BMP) is a member of the TGF-β superfamily, and is a strong bone/cartilage inductive molecule *in vivo* and *in vitro*[8,9]. Previous immunohistochemical and *in situ* hybridization studies clarified that BMP is induced and localized in cells under various osteochondrogenic conditions, suggesting the involvement of BMP in osteo/chondro inductive events *in vivo*[9,10]. Furthermore, we recently reported that several BMP genes are localized at the site of chondrogenesis in a mice spondylosis model that histologically resembles the degenerative process of cartilage in humans[11]. To date, almost 30 members of the BMP family have been identified[9], and among its members, BMP-2 has the capacity to induce osteochondrogenesis[3,9], and reportedly promotes growth and matrix synthesis of chondrocytes[5,12]. This chondrogenic action of BMP-2 easily leads us to question the involvement and role of this molecule in OA. However, to date, there have been no reports of the expression and localization of BMP-2 in OA tissue.

The purpose of this study was to elucidate the localization of BMP-2 mRNA and protein in surgical OA specimens and in normal cartilage using immunohistochemistry and *in situ* hybridization.

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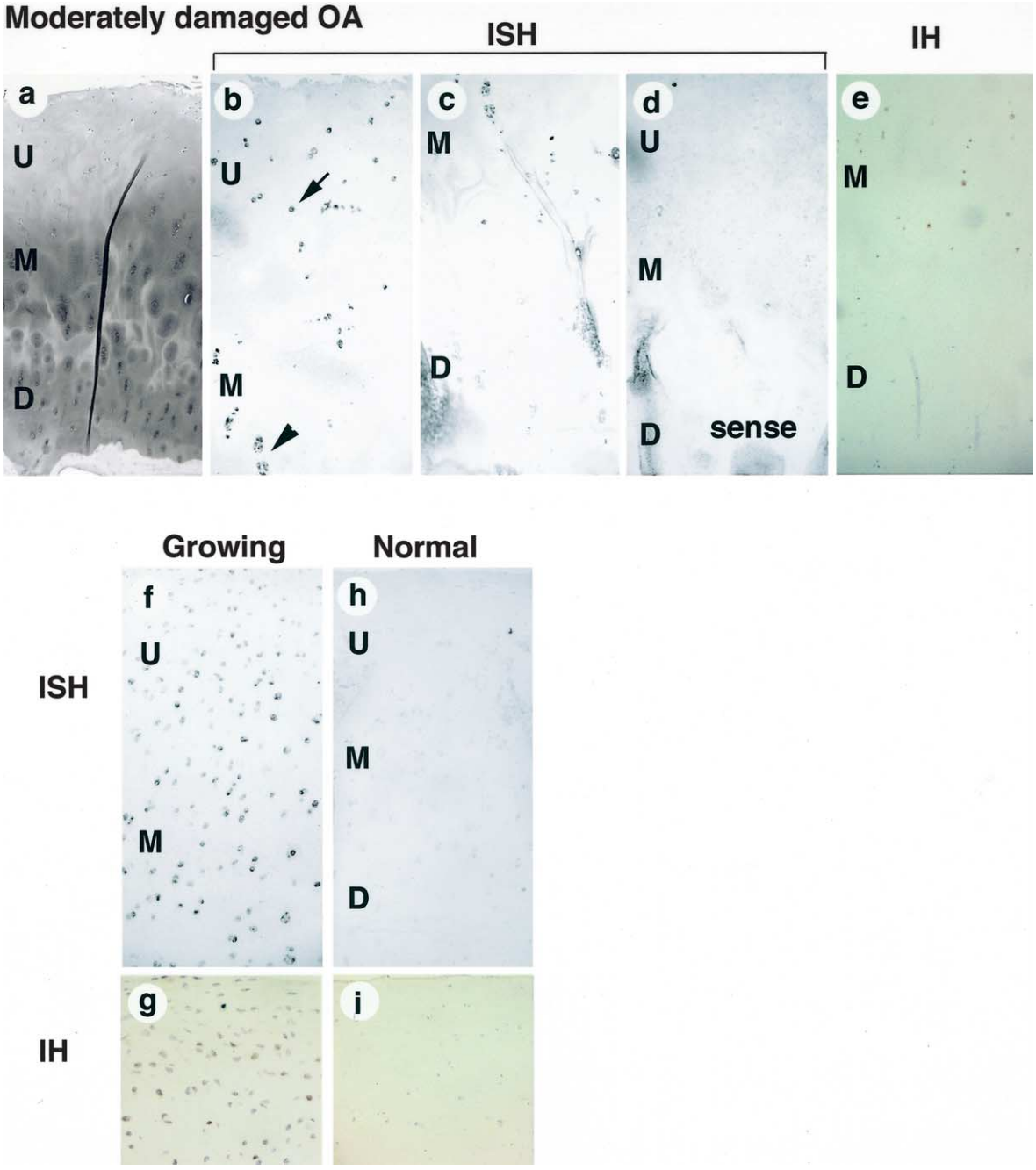


Fig. 1. Histological sections of moderately damaged OA (a–e; Mankin grade 3), growing (f, g) and normal articular cartilage (h, i). Safranin-O/fast green staining (a); *in situ* hybridization (ISH) with antisense (b, c, f, h) and with sense (d) human BMP-2 cRNA probe; and immunohistochemistry (IH) with anti-BMP-2/4 antibody (e, g, i). (U, upper zone; M, middle zone; D, deep zone; growing, growing neonatal cartilage; normal, normal adult cartilage) (a) Safranin-O staining was decreased in the upper layer, but preserved in the middle and deep layers. (b, c) BMP-2 mRNA was detected in individual (arrow) and clustering (arrow head) chondrocytes in the upper and middle layers (b). In contrast, BMP-2 mRNA was not detected in deep zone cells (c). (d) No signals were detected with sense probe (continuous section from a). (e) BMP-2/4 was immunolocalized in chondrocytes in the middle zone (M). In contrast, immunoreactivity was not apparent in deep zone (D) chondrocytes. (f, g) BMP-2 transcripts (f) and positive immunostaining for BMP-2/4 (g) were observed in articular chondrocytes in human neonatal growing articular cartilage (ankle joint). (h, i) BMP-2 transcripts (h) and positive immunostaining for BMP-2/4 (i) were not detected in cells in normal adult articular cartilage (knee joint). Original magnification: ×40 (a, d, f, h); ×100 (b, c, e); and ×200 (g, i).

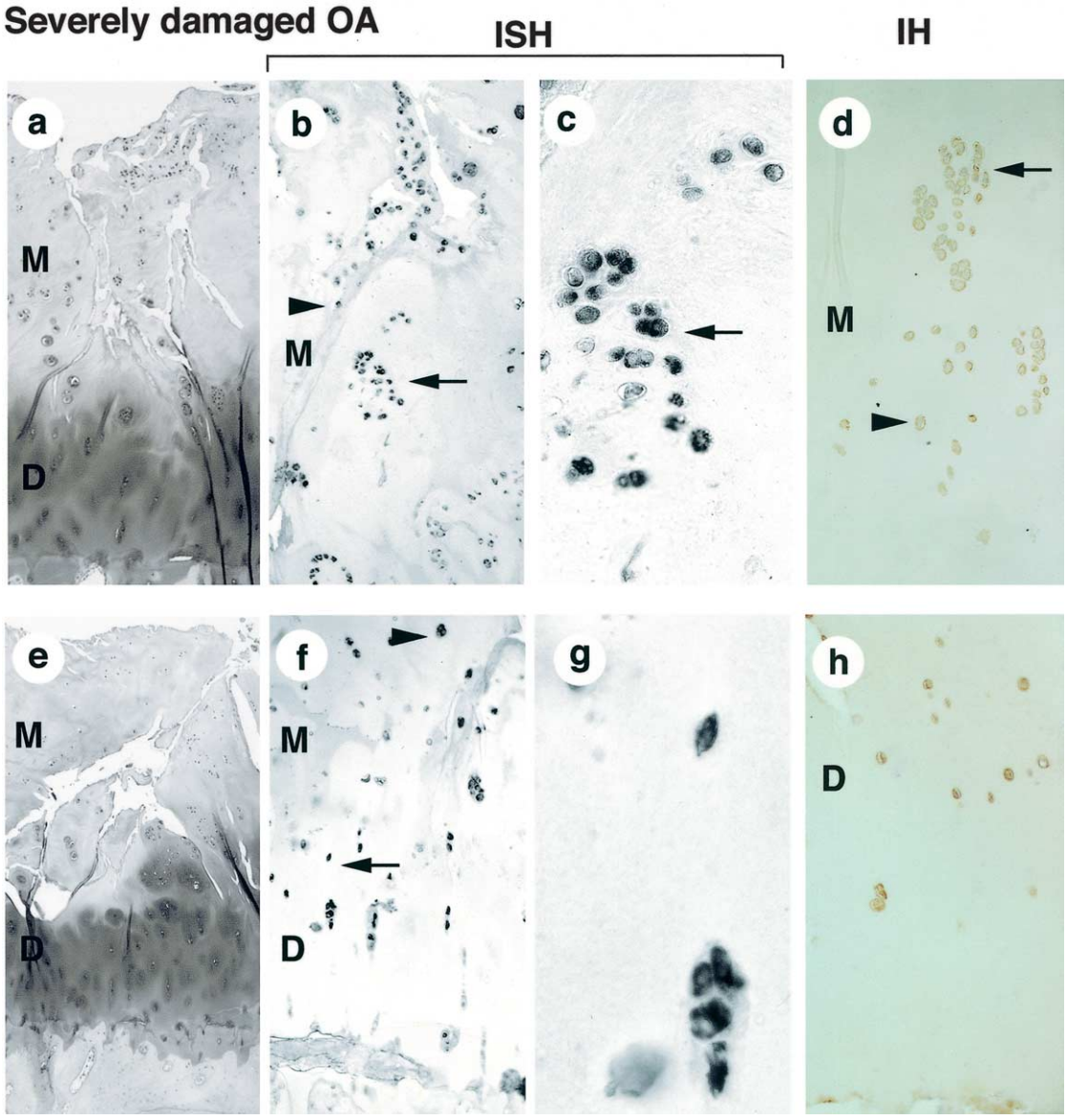


Fig. 2. Histological sections of severely damaged OA cartilage (Mankin grade 9). Staining of safranin-O/fast green (a, e); *in situ* hybridization (ISH) with antisense human BMP-2 cRNA probe (b, c, f, g); and immunohistochemistry (IH) with anti-BMP-2/4 antibody (d, h). (U, upper zone; M, middle zone; D, deep zone) (a) Upper zone was essentially lost and staining for safranin-O was lacking in the middle zone. (b) Intense signals for BMP-2 transcripts were detected in both individual (arrow head) and clustering chondrocytes (arrows) in the middle layer in which the territorial matrix was less stained by safranin-O. (c) Higher magnification of arrow area in (b) in the middle zone. BMP-2 mRNA was detected in clustering chondrocytes. (d) Positive immunostaining for BMP-2/4 was detected both in the individual (arrow head) and clustering (arrow) chondrocytes in the middle layer (nearby section to a). (e) Upper zone was essentially lost and positive staining for safranin-O was apparent in the territorial matrix of the deep zone chondrocytes. (f) BMP-2 mRNA was localized in middle layer chondrocytes (arrow head). BMP-2 mRNA was also detected in both individual and clustering chondrocytes in the deep layer (arrow), whose territorial matrix was stained by safranin-O. (g) Higher magnification of arrow area in (f). BMP-2 mRNA was detected the deep layer chondrocytes. (h) BMP-2/4 was also immunolocalized in deep layer chondrocytes. Original magnification: ×40 (a, e); ×100 (b, f); ×200 (d, h); and ×400 (c, g).

Materials and methods

TISSUE SAMPLING AND PREPARATION

Cartilage specimens with subchondral bone were obtained from 10 female and four male patients (age range, 65 to 75 years) undergoing endoprosthetic surgery for OA of the knee joints (medial and lateral femoral condyles and tibial articular surfaces). All the cases satisfied the American College of Rheumatology classification criteria for OA of the knee[13]. Cases of rheumatoid arthritis were excluded. Five macroscopically normal cartilage specimens with subchondral bone were obtained from five patients undergoing amputation (age range, 36 to 66 years; two medial femoral condyles, two tali of ankle joint and one metatarsal head of interphalangeal joints). In addition, four growing articular cartilage samples were obtained from four patients undergoing amputation (age range, 1 to 4 years; two tali and two metatarsal heads). Written, informed

Table I
Summary of BMP-2 mRNA localization in cells in moderately and severely damaged OA cartilage

Degree of damage	Type of chondrocytes	Zone		
		Upper	Middle	Deep
Moderate	Individual	+~++	+~++	−~±
	Clustering	+~+++	++~+++	−~+
Severe	Individual	*	++~+++	++~+++
	Clustering	*	++~+++	+++

Numbers of individual and clustering chondrocytes positive for BMP-2 mRNA were counted. Ratio indicates positive individual chondrocytes/total individual chondrocytes, and positive clustering chondrocytes/total clustering chondrocytes.
−, 0%; ±, ~10%; +, ~25%; ++, 25–50%; +++, 50–75%.
*Upper zone was almost lost.

consent was obtained from all the patients or legal guardians. Obtained tissues were fixed in 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) in PBS (pH, 7.4) (Sigma Chemical Co., St. Louis, MO) at 37°C for 24 h, decalcified in 20% EDTA (if needed) and dehydrated in an ethanol series before being embedded in paraffin as described[10,14]. The size of each section ranged from approximately 0.8×1 to 1.5×2.8 cm. Each 5-μm section was made on a microtome, and the sections were stained with safranin-O/fast green to reveal cartilaginous matrix. Three sections from each specimen were used for immunohistochemistry and *in situ* hybridization. The histological samples were graded according to the method of Mankin *et al.*[15].

PREPARATION OF RNA PROBES

cDNA for human BMP-2[16] (encoding the propeptide region relatively specific for BMP-2; with less than 54% homology with human BMP-4 and less than 33% homology with human BMP-6 and BMP-7) was subcloned into a pGEM-T plasmid, and then either linealized by Sac II and transcribed by SP6 RNA polymerase to generate antisense probes or linealized by Spe I and transcribed by T3 RNA polymerase to generate sense probes as described[16]. Northern blotting experiments using ^{32}P-labeled antisense transcripts and the human osteosarcoma cell line showed a specific band (data not shown). The antisense probe for 18S ribosomal RNA (rRNA) was prepared as described[3] and was used to confirm the preservation of RNA in the tissue specimens.

IN SITU HYBRIDIZATION

In situ hybridization was performed as previously described[10,16]. Briefly, paraffin-embedded sections were dewaxed, rehydrated, and fixed with 4% paraformaldehyde for 20 min. The sections were then treated with 0.2 N HCl for 10 min to inactivate endogenous alkaline phosphatase, and were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH, 8.0) for 10 min. They were then dehydrated with an ethanol series and were air-dried. Each section was covered by 50 μl of hybridization solution (50% formamide, 10% dextran sulfate, 1×Denhardt's solution, 600 mM NaCl, 0.25% SDS, 150 μg/ml of *Escherichia coli* tRNA, approximately 0.5 μg/ml of RNA probe) and incubated at 50°C for 16 h. After hybridization, the slides were briefly washed in 5×SSC (1×SSC=0.15 M NaCl, 0.015 M sodium citrate) and in 50% formamide, 2×SSC for 30 min at 50°C. RNase A treatment (10 μg/ml) proceeded at 37°C for 30 min. The slides were washed twice with 2×SSC and 0.2×SSC for 20 min at 50°C. Hybridized probes were detected using a Nucleic Acid Detection Kit (Boehringer Mannheim GmbH Biochemica, Mannheim, FRG) according to the manufacturer's instructions.

Controls included: (i) hybridization with the sense (mRNA) probe, (ii) RNAse A treatment (20 μg/ml) prior to hybridization, and (iii) use of neither antisense nor anti-digoxigenin antibody. The three controls showed no positive signals. The cell count assessment for positive cells was done at ×200 magnification using a NIKON ECRIPS E800M microscope (Nikon, Tokyo, Japan) as described[11].

IMMUNOHISTOCHEMISTRY

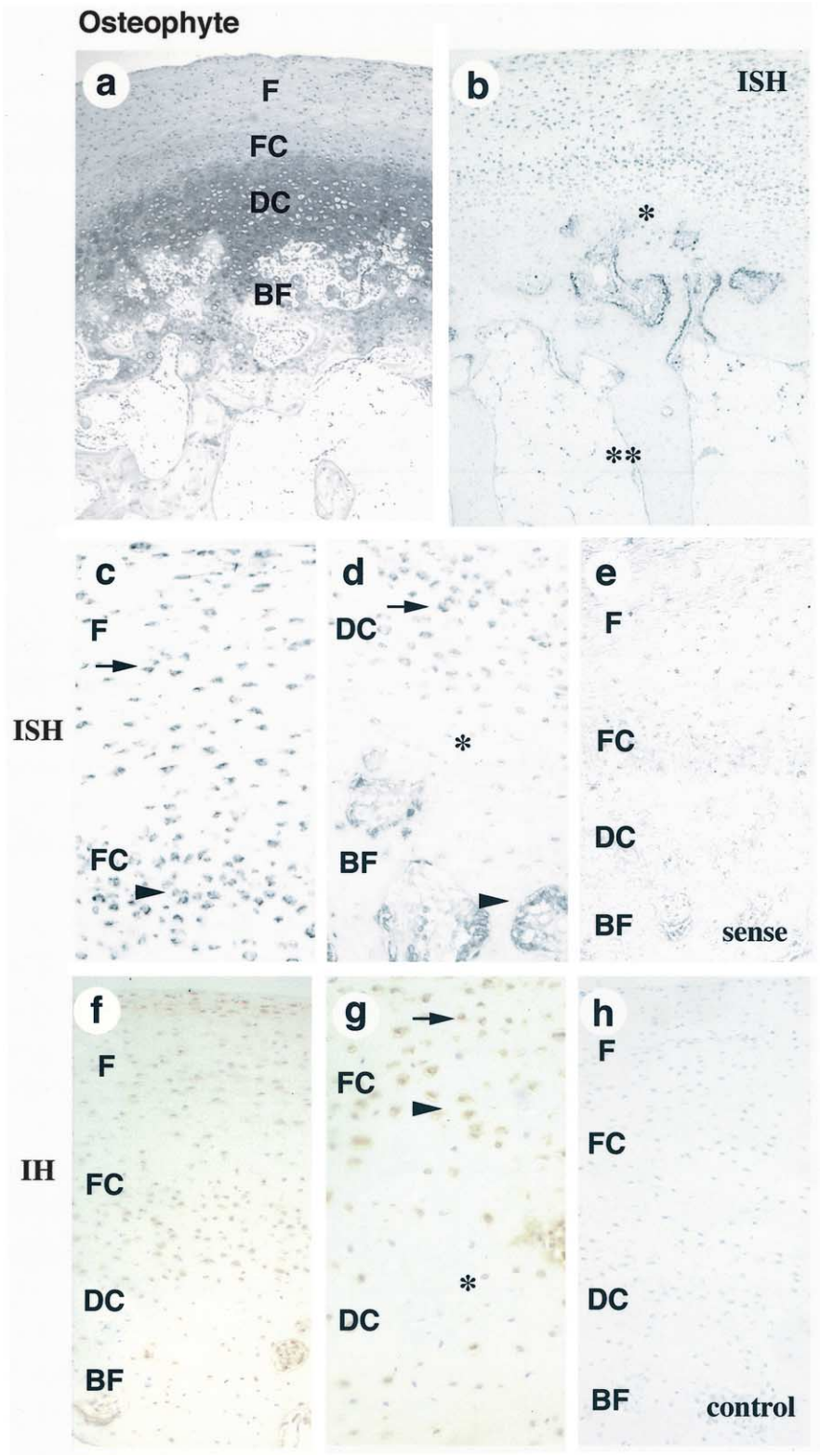
Immunohistochemistry was performed using the streptoavidin–peroxidase method with histofine SAB-PO kits (Nichirei, Tokyo, Japan) as described with minor modification[12]. A mouse monoclonal antibody against human BMP-2/4 (h3b2/17.8.1, kindly donated by Genetics Institute, Inc., MA)[14,15] was used as a primary antibody at a concentration of 10 μg/ml. Among several BMP members (BMPs 1 to 7), only BMP-2 and -4 were found to react with this antibody by immunoblotting with purified recombinant proteins[17]. Tissue sections were briefly deparaffinized and were incubated with 2 mg/ml hyaluronidase in phosphate buffered saline (PBS; pH, 7.5) for 20 min. The sections were digested with 1 mg/ml pronase (Boehringer Mannheim GmbH Biochemica, Mannheim, FRG) in PBS (pH, 7.5) and placed in 3% H_2O_2 in methanol for 30 min to block endogenous peroxidase. After washing in PBS, the sections were blocked with 10% normal serum of the same species as the secondary antibody to minimize background staining, before being incubated with primary antibody for 2 h at room temperature. Normal serum of the same species as the primary antibody was used as a control for the primary antibody. After washing in PBS, the sections were incubated with secondary antibody at a concentration of 1:100 (rabbit anti-mouse Ig-G: Nichirei) for 20 min at room temperature in a humid chamber, and then incubated with peroxidase-conjugated streptoavidin (Nichirei), again for 20 min at room temperature in a humid chamber and washed in PBS. Finally, color reaction was performed using the substrate reagent 3,3′diaminobenzidine tetrahydrochloride (DAB) (Dojindo, Tokyo, Japan). This final solution contained 120 ml of Tris–HCl (pH, 7.6), 15 mg of DAB and 6.8 μl of 35% H_2O_2. Sections were counterstained with hematoxylin and mounted. As a control for immunostaining, normal serum from the same species as the primary antibody (mouse-Ig-G) was used.

Results

BMP-2 LOCALIZATION IN OA CARTILAGE

In situ hybridization detected BMP-2 mRNA in cells at various Mankin grades in OA cartilage (Figs. 1 and 2). BMP-2 mRNAs were localized in both clustering and individual chondrocytes.

In moderately damaged OA cartilage (Mankin grade 3–6; Fig. 1), staining of safranin-O was still preserved in the middle and deep layers [Fig. 1(a)]. BMP-2 mRNA was localized in chondrocytes in both the upper and middle layers [Fig. 1(b)]. In contrast, BMP-2 transcript was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scarcely detected in deep layer chondrocytes [Fig. 1(c)], although these cells were positive for 18S rRNA (data not shown). No signals were detected with BMP-2 sense probe [Fig. 1(d)]. BMP-2/4 was immunolocalized in the upper layer (data not shown) and middle layer chondrocytes, but was scarcely immunolocalized in deep layer chondrocytes [Fig. 1(e)].

BMP-2 mRNA and BMP-2/4 were localized in neonatal growing articular chondrocytes [Fig. 1(f,g)]. In contrast, BMP-2 mRNA was absent and BMP-2/4 was not localized in normal adult cartilage cells [Fig. 1(h,i)].

In severely damaged OA cartilage (Mankin grade 7–12; Fig. 2), staining intensity of safranin-O noticeably decreased. BMP-2 transcripts were detected not only in middle zone chondrocytes [Fig. 2(a–c)], but also in deep zone chondrocytes [Fig. 2(e–g)]. In the middle layer, BMP-2 transcripts were located in clustering chondrocytes whose territorial matrix lacked safranin-O staining [Fig. 2(a–c)]. In the deep layer, BMP-2 mRNA was localized in chondrocytes whose territorial matrix was stained with safranin-O [Fig. 2(e–g)]. BMP-2/4 was immunolocalized both in the middle and deep layer chondrocytes [Fig. 2(d,h)], and was immunolocalized in both individual and clustering chondrocytes [Fig. 2(d,h)].

The pattern of BMP-2 mRNA localization in OA cartilage is summarized in Table I. The pattern of BMP-2/4 immunolocalization was associated with that of mRNA localization.

BMP-2 LOCALIZATION IN OSTEOPHYTIC TISSUE

BMP-2 mRNA was intensely localized in cells in osteophytic tissue [Fig. 3(a–d)]. BMP-2 transcripts were localized in fibroblastic cells in the outer fibrous layer [Fig. 3(b,c)], in fibrochondrocytes in the fibrocartilage layer [Fig. 3(b,d)] and in osteoblasts in the bone-forming area adjacent to the chondrogenic layer [Fig. 3(b,d)]. The majority of chondrocytes in the deep cartilage layer were positive for BMP-2. However, chondrocytes in the very deep layer close to the bone-forming site showed no, or only faint, signals for BMP-2 transcripts [Fig. 3(b,d)]. BMP-2 transcripts were absent in osteoblasts located in the relatively mature bone at the center of the osteophyte [Fig. 3(b)]. No signals were detected with the BMP-2 sense probe [Fig. 3(e)]. BMP-2/4 immunolocalization was associated with mRNA localization [Fig. 3(f,g)]. Negative control using normal mouse serum showed no staining [Fig. 3(h)].

Discussion

To the best of our knowledge, this is the first report to describe the localization of BMP-2 in human OA tissues. BMP-2 was scarcely detected in adult normal cartilage, but was notably present in the OA cells. These findings indicate the possible synthesis of BMP-2 by OA cells and involvement of BMP-2 in the OA state. BMP-2 was synthesized by both individual and clustering chondrocytes. Even deep zone chondrocytes synthesized BMP-2 in severe OA cartilage, where cartilage damage was extended to the deeper area, although these cells did not synthesize BMP-2 when cartilage damage was less significant. These findings suggest that the zone-specific distribution of BMP-2 may be dependent on the degree of OA damage.

Our findings of this study, as well as those of previous reports[3,18], indicate that some of the BMP-2 positive cells are associated with proteoglycan synthesis, as evident by positive staining for safranin-O in the territorial matrix. This tendency was particularly marked in the lower middle and deep layers. OA chondrocytes in the middle and deep layers reportedly synthesize several cartilaginous collagens[3] and aggrecan mRNA[18]. Another study has reported that BMP-2 stimulates the synthesis of proteoglycan synthesis in articular cartilage[5]. Given our results and those of previous reports, BMP-2 localization in safranin-O positive areas suggests the possible involvement of BMP-2 in neo-matrix synthesis occurring in OA tissue. We found clustering chondrocytes whose territorial matrix lacked safranin-O staining. These types of cells are thought to be in a proliferating state[2]. BMP-2 was localized in these types of cells and given that BMP-2 reportedly promoted growth of chondrocytes in *in vitro* culture experiments[12], BMP-2 may be involved in OA cell proliferation as well as matrix synthesis.

Significant BMP-2 expression was observed in osteophytic tissue. BMP-2 was detected in the early mesenchymal layers as well as in fibrochondrocytes, suggesting the role of BMP-2 in induction and promotion of osteophyte formation. Such osteochondro-inductive action of BMP-2 has been proposed by previous *in vivo* and *in vitro* studies[8,9]. In the present study, the localization of BMP-2 in OA chondrocytes at various Mankin grades in various zones and in fibrochondrocytes in the osteophyte is somewhat similar to that of Col IIA[4]. As Col IIA is reportedly a marker for chondroprogenitor cells and binds to BMP-2, BMP-2 may be involved in the early chondrogenic events via the BMP-2/Col IIA complex, leading to further matrix synthesis in OA cartilage and osteophyte formation.

As BMP-2/4 could not be detected in the extracellular matrix (ECM) in OA cartilage during the present experiments, but was localized in OA cells, it is possible that the amount of BMP-2/4 in ECM is considerably small, or the lack of BMP-2/4 localization in the ECM might be due to the technical limitations of the sensitivity of our

Fig. 3. (a) Staining of safranin-O/fast green; *in situ* hybridization (ISH) (b–d) with antisense and (e) sense human BMP-2 cRNA probes; and (f, g) immunohistochemistry (IH) with anti-BMP-2/4 antibody and (g) normal mouse serum in histological sections of human osteophytic tissue. (a, b) BMP-2 mRNA was detected in fibroblastic mesenchymal cells in the outer fibrous layer (F), in fibrochondrocytes in the middle fibrocartilage layer (FC) and in chondrocytes in the deep chondrocyte layer (DC). In contrast, BMP-2 transcript was absent in cells in the very deep zone [asterisk in (b)] of the deep cartilage layer close to the bone-forming area. BMP-2 signals were present in osteoblasts in the bone-forming layer (BF), but absent in cells in mature bone located at the center [double asterisks in (b); (a, b, e, f) are nearby sections]. (c) Higher magnification of the F and FC layers shown in (b). Intense signals for BMP-2 mRNA were detected in fibroblastic cells (arrow) and fibrochondrocytes (arrow head). (d) Higher magnification of the DC and the BF layers is presented in (b). BMP-2 transcripts were detected in chondrocytes (arrow) and osteoblasts (arrow head). In contrast, no, or faint, signals for BMP-2 mRNA were detected in the very deep zone chondrocytes (asterisk) close to the BF area. (e) No apparent signals were detected in any layer by human BMP-2 sense probe. Positive BMP-2/4 staining was observed in cells in the outer fibrous (F) layer. (f, g) Positive immunostaining for BMP-2/4 was detected in cells in the outer fibrous layer (F) (f), the middle fibrocartilage layer (FC) (f and arrow in g), and in chondrocytes in the deep chondrocytes layer (DC) (g and arrow head in g). (h) Negative control. No immunostaining was observed with mouse normal serum. Original magnification: ×40 (a, b); ×100 (e, f, h); and ×200 (c, d, g).

immunohistochemical methods. These possibilities should be further investigated. In addition, several other growth factors, such as TGF-β[7] insulin-like growth factor[19], basic fibroblast growth factor[7], hepatocyte growth factor[20], vascular endothelial cell growth factor[21] and cartilage-derived morphogenetic protein-1[22], are reported to be expressed in OA cartilage. BMP-2 may act synergistically with these growth factors or share roles in the metabolism of OA cartilage. A recent publication, in particular, has proved the potent role of TGF-β in osteophyte formation in an experimental animal model[6]. Future studies should aim to elucidate the precise roles of BMP-2 in OA change.

In conclusion, this is the first report to demonstrate BMP-2 localization in OA tissue. The present findings, together with those of previous reports, suggest the anabolic role of OA chondrocytes via synthesis of BMP-2 and the potential capacity of articular chondrocytes to synthesize BMP-2 in response to the cartilage damage that occurs in osteoarthritis.

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