

Expression of Bone Morphogenetic Protein 6 in Healthy and Osteoarthritic Human Articular Chondrocytes and Stimulation of Matrix Synthesis In Vitro

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Objective. To elucidate the role of bone morphogenetic protein 6 (BMP-6) in human articular cartilage, we investigated whether BMP-6 is expressed in adult human articular chondrocytes and analyzed the potential stimulatory effects of BMP-6 on these cells. In addition, we investigated whether osteoarthritic (OA) and normal cartilage chondrocytes behave differently.

Methods. Endogenous expression of the BMP-6 gene was examined by reverse transcription–polymerase chain reaction. BMP-6 protein was detected by Western immunoblotting. Chondrocytes were grown as monolayer cultures for 7 days in a chemically defined serum-free medium, in the absence or presence of recombinant BMP-6. Proteoglycan (PG) synthesis was measured by ³⁵S-sulfate incorporation into newly synthesized macromolecules. Cell proliferation was assessed by ³H-thymidine incorporation.

Results. BMP-6 was expressed in both healthy and OA chondrocytes at the messenger RNA and protein levels. Total PG synthesis was significantly increased after BMP-6 stimulation of healthy (mean ± SEM 191 ± 11%; *P* < 0.001) and OA (150 ± 25%; *P* < 0.03) chondrocyte cultures. A direct comparison between healthy and OA samples revealed no significant difference. The proliferation rates of normal and OA chondrocytes were not affected by BMP-6 treatment.

Conclusion. BMP-6 is endogenously expressed in chondrocytes obtained from OA and normal adult human articular cartilage. Furthermore, BMP-6 has the potential to stimulate total PG synthesis in human articular chondrocytes derived from normal as well as OA joints. We conclude that the presence of BMP-6 in adult human articular cartilage indicates a functional role for this growth factor in the maintenance of joint integrity.

To effectively maintain the integrity of articular cartilage, a constant synthesis of matrix macromolecules by articular chondrocytes is essential. The biosynthetic activity of articular chondrocytes depends, at least in part, on the ability to respond to anabolic growth factors. In recent years, numerous regulators of skeletal development have been identified, including members of the bone morphogenetic protein (BMP) family. BMPs are characterized by their unique ability to induce endochondral bone formation when implanted at ectopic sites (1,2). They belong to the large transforming growth factor β (TGF β) superfamily of secreted signaling molecules (3). BMP-6 (also known as vegetal related 1) belongs to a subgroup of BMPs that includes BMP-5, BMP-10, osteogenic protein 1 (OP-1), OP-2, OP-3, and growth/differentiation factors 2 and 11 (4). BMP-6 is expressed in a variety of embryonic, neonatal, and adult tissues, such as the epidermis, central nervous system, lung, and kidney (5–7). Moreover, it is known to be actively involved in chondrocyte metabolism, since it promotes chondrocyte differentiation in the growth plate (8) and in human marrow stromal cells (9). However, little is known about the role of BMP-6 in adult cartilage.

Given the role of BMP-6 in chondrocyte development, we investigated the endogenous expression of BMP-6 and examined its potential in maintaining

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cartilage homeostasis. Our data demonstrated the presence of BMP-6 in normal as well as osteoarthritic (OA) adult human articular cartilage. Furthermore, BMP-6 stimulated total proteoglycan (PG) synthesis in healthy and OA chondrocytes, suggesting a role in cartilage maintenance and repair, as well as a therapeutic potential.

PATIENTS AND METHODS

Cell culture. Human articular cartilage without macroscopic evidence of tissue damage was obtained from the femoral heads of 16 patients (ages 37–80 years, mean age 58.1 years) at the time of endoprosthetic replacement for acute transcervical fractures and from 11 organ donors (ages 37–60 years, mean age 50.5 years) within 12 hours of death. The removal of cartilage from organ donors was approved by the Ethics Committee of the University of Vienna.

Cartilage samples were also obtained from 12 femoral heads from patients with hip OA at the time of surgery for total hip endoprosthesis. The age of the OA patients ranged from 35 to 84 years (mean age 58.6 years). Macroscopically intact as well as fibrillated cartilage was obtained from the OA specimens. Dissection of neocartilage at the joint margins was avoided.

Cartilage slices were dissected aseptically from the total surface of the femoral head and then finely minced. Chondrocytes were released by overnight digestion in 0.2% collagenase B (Boehringer Mannheim, Mannheim, Germany) and filtered through a cell strainer (Falcon Labware; Becton Dickinson, Lincoln Park, NJ) to remove debris and undissociated cell clusters. The cell filtrate was then centrifuged at 500g for 10 minutes. Pellets were resuspended in a 1:1 mixture of Dulbecco's modified Eagle's medium (25 mM HEPES, 4,500 mg/liter of glucose, and pyridoxine, without sodium pyruvate; Life Technologies, Gaithersburg, MD) and Ham's F-12 (Ham's F-12 plus L-glutamine; Life Technologies) containing 10% fetal bovine serum (FBS; PAA Laboratories, Linz, Austria) and antibiotics/antimycotics (100 units/ml of penicillin G, 100 mg/ml of streptomycin, and 0.25 μ g/ml of amphotericin B; Life Technologies). Evaluation of the chondrocyte number was done after Trypan blue staining in a Buerker-Tuerk chamber.

Cells isolated from healthy specimens were grown as monolayer cultures in 24-well multiwell plates (Costar, Cambridge, MA), in quadruplicate, at a density of 1×10^5 cells/cm² and were used for ³⁵S-sulfate incorporation and Western blot analysis. For cell proliferation, chondrocytes were cultured in 96-well multiwell plates (Packard, Meriden, CT) at a density of 5,000/well. For RNA extraction, 2×10^6 chondrocytes were seeded in 100-mm tissue culture dishes (Costar), and at 90% confluence, serum-containing medium was changed to a chemically defined serum-free basal medium (BM), as described elsewhere (10). The cells were subsequently cultured with and without recombinant BMP-6 (final concentration 100 ng/ml) for 24 hours (cell proliferation experiments) or for 7 days (PG synthesis and RNA extraction). Cultures treated with 10% FBS were run in parallel as positive controls. The medium and the recombinant growth factor were replaced every other day.

Cultures were maintained at 37°C in an atmosphere of humidified air and 5% CO₂.

Growth factor. The recombinant growth factor BMP-6 was kindly provided by Stryker Biotech, Hopkinton, MA. Aliquots were stored at –80°C until used.

Cell proliferation. Chondrocytes obtained from 3 healthy and 4 OA specimens were preincubated with serum-free medium for 24 hours. BMP-6 was added for an additional 24 hours. Cultures treated with 10% FBS were run in parallel as positive controls. Cells were pulsed with 1 μ Ci/ml of methyl-³H-thymidine (Amersham, Buckinghamshire, UK) for the last 6 hours of culture. The cells were washed extensively with phosphate buffered saline (PBS; Life Technologies), and ³H-thymidine incorporation in the culture plate was measured in a liquid scintillation counter (Packard), after the addition of scintillant.

Biosynthesis of macromolecules. To study the stimulatory effects of BMP-6 on total PG synthesis, ³⁵S-sulfate incorporation into sulfated glycosaminoglycans was performed as described previously (11). Briefly, cells were radiolabeled by incubation with 20 μ Ci/ml of ³⁵S-sulfate (carrier-free; Amersham) for 6 hours at 37°C in BM. Chondrocytes were extracted in guanidine HCl buffer (4M guanidine HCl, 50 mM sodium acetate, pH 7.2, in the presence of protease inhibitors). Unincorporated isotope was removed by using Sephadex G-25 gel chromatography (PD-10 columns; Pharmacia Biotech, Piscataway, NJ). Values were obtained by liquid scintillation counting (model 1410 liquid scintillation counter; Wallac, Turku, Finland) of aliquots from void volume fractions and were normalized to the protein content. Total cellular protein was determined by the Bradford method, using a Bio-Rad Protein Assay kit according to the manufacturer's instructions (Bio-Rad, Munich, Germany).

RNA isolation and Northern blot analysis. Total RNA was extracted using the modified acid guanidine-phenol-chloroform method established by Chomczynski and Sacchi (12). For Northern blot analysis, equal amounts (5 μ g) of total RNA, determined by absorbance at 260 nm, were electrophoresed on 1.2% agarose-formaldehyde gels and transferred onto Nytran membranes (Schleicher & Schuell, Keene, NH). After ultraviolet cross-linking, the blots were prehybridized at 68°C for 30 minutes in hybridization buffer (ExpressHyb; Clontech, Palo Alto, CA). Hybridization was performed in the presence of a ³²P-radiolabeled complementary DNA (cDNA) probe for 1 hour at 68°C. Fragments of cDNA from the primary PG of cartilage matrix aggrecan (forward 5'-CGC-TAC-GAC-GCC-ATC-TGC-TAC-3' and reverse 5'-GCC-TGC-TGT-GCC-TCC-TCA-AA-3') (13) and β -actin (forward 5'-TGT-GAT-GGT-GGG-AAT-GGG-TCA-G-3' and reverse 5'-TTT-GAT-GTC-ACG-CAC-GAT-TTC-C-3') were amplified by reverse transcription-polymerase chain reaction (RT-PCR), purified, and labeled with α -³²P-dCTP (Amersham), using a random hexanucleotide-primed second-strand synthesis method. Membranes were subsequently washed 3 times in 2 \times saline-sodium citrate (SSC), 0.05% sodium dodecyl sulfate (SDS), then twice in 0.2 \times SSC, 0.1% SDS, at room temperature for 10 minutes and then exposed to Kodak XAR-5 film at –70°C for up to 24 hours.

Analysis of the endogenous expression of BMP-6 by RT-PCR. RT-PCR was used to determine the presence of BMP-6 at the messenger RNA (mRNA) level. Total RNA

from unstimulated chondrocyte cultures was extracted as described for Northern analysis, and 1 μ g of each sample was copied into cDNA in a 20- μ l reaction using a First-Strand cDNA Synthesis kit (Pharmacia, Uppsala, Sweden). One-microliter aliquots were amplified in a 10- μ l reaction mixture that contained 50 mM Tris HCl (pH 8.3), 2 mM MgCl₂, 0.25% bovine serum albumin, 2.5% Ficoll 400, 5 mM tartrazine, 200 μ M of dNTP, 1 μ M of each primer, and 0.2 units of *Taq* polymerase (Boehringer Mannheim). Primer sequences for BMP-6 were as follows: forward 5'-CAG-GAG-CAT-CAG-CAC-AGA-GA-3' and reverse 5'-ATG-TGT-GCG-TTG-AGT-GGG-AA-3'. The following reaction profile was used for all experiments: an initial denaturation at 94°C for 1 minute, followed by 30 cycles at 94°C for 1 second, 52°C for 1 second, and 72°C for 40 seconds, and an additional 2-minute extension step at 72°C after the last cycle. Amplification reactions were performed in an air thermal cycler (Idaho Technology, Idaho Falls, ID).

Reaction products were analyzed by electrophoresis using 1.5% agarose gels. The amplified DNA fragments were stained with SYBR Green (Molecular Probes, Eugene, OR) and visualized with a FluorImager (Molecular Dynamics, Sunnyvale, CA). Negative controls in which cDNA was omitted from the reaction were run in parallel.

Analysis of the endogenous expression of BMP-6 by Western immunoblotting. To prove the presence of BMP-6 protein in healthy and OA cartilage, Western immunoblots of tissue extracts and cell lysates were performed. For Western blot analysis of cell lysates, chondrocytes from 5 healthy and 5 OA cartilage samples were isolated and cultured in 24-well multiwell plates (Costar) at a density of 2×10^5 cells/well, as described above, until subconfluence was reached. The wells were then washed 3 times with PBS, and 50 μ l of sample buffer (4 \times NuPAGE LDS sample buffer; Invitrogen, Carlsbad, CA) was added to each well. Cells were disrupted by ultrasound, and samples were stored at -80°C until used.

For analysis of cartilage extracts, slices of freshly isolated articular cartilage (0.5–1 gm wet weight) were washed with PBS, finely minced, and extracted in guanidine HCl buffer (1M guanidine HCl, 50 mM sodium acetate buffer, pH 7.2) in the presence of protease inhibitors for 3 hours at 4°C. Samples were then dialyzed (Slide-A-Lyzer dialysis cassette; Pierce, Rockford, IL) against water for 48 hours at 4°C. Extracts were then lyophilized, resuspended in sample buffer (NuPAGE LDS sample buffer) to which 8M urea was added, and stored at -80°C.

Aliquots of the samples (1 μ l per lane) were analyzed under reducing conditions (2% β -mercaptoethanol). Samples were boiled for 5 minutes in a water bath at 95°C and loaded onto a 10% Bis-Tris gel (NuPAGE 10% Bis-Tris Gel; Invitrogen). Electrophoresis was performed using a commercially available running buffer (20 \times NuPAGE MOPS SDS running buffer; Invitrogen) at 200V for 60 minutes. Proteins were transferred from the gel onto a nitrocellulose membrane for 120 minutes at 30V in a buffer containing 0.4M glycine, 0.5M Tris base, and 0.01M SDS. Nonspecific binding sites were blocked with 4% bovine serum albumin for 60 minutes. Blots were then probed with a polyclonal antibody against BMP-6 (R&D Systems, Minneapolis, MN) and thereafter incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA)

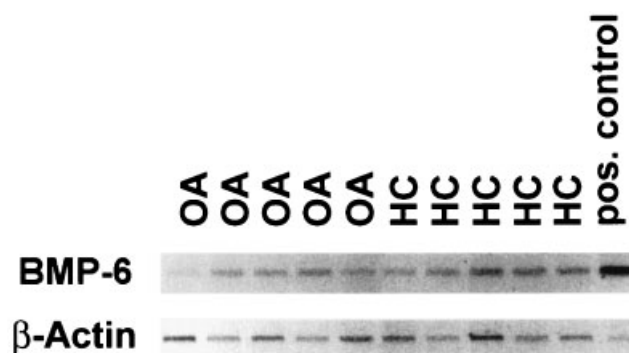


Figure 1. Endogenous expression of bone morphogenetic protein 6 (BMP-6) in human chondrocytes derived from healthy control (HC) and osteoarthritic (OA) joints. Randomly selected cartilage samples from 5 OA and 5 normal joints were used. Cells were cultured in serum-free basal medium for 7 days. Then, cDNA was prepared as described in Patients and Methods and subjected to polymerase chain reaction (PCR) amplification using a primer sequence for BMP-6. The PCR mixture were separated on a 1.5% agarose gel, stained with SYBR Green, and visualized on a FluorImager system.

at a dilution of 1:1,000. For detection enhancement, 1 ml of a chemiluminescent substrate (SuperSignal West Pico chemiluminescent substrate; Pierce) was applied to the membrane. Recombinant human BMP-6 and BMP-7 were used as positive controls; sample buffer served as a negative control. Blots were visualized on chemiluminescence imaging film (Hyperfilm; Amersham).

Statistical analysis. Statistical analysis was performed using Student's *t*-test. A normality test was performed to determine whether the distribution of the samples was Gaussian. To examine the relationships between age and PG synthesis rate, Pearson's correlation calculations were used. *P* values less than 0.05 were considered significant.

RESULTS

Endogenous expression of BMP-6 mRNA in human articular chondrocytes. While healthy and OA adult human articular chondrocytes endogenously express receptors for BMPs 1 and 2 as well as several members of the BMP family of growth factors (14,15), it is unknown whether BMP-6 is also expressed. To investigate the expression of BMP-6, we performed RT-PCR of total RNA obtained from the cartilage of 10 randomly selected subjects: 5 with (ages 35–80 years) and 5 without (ages 39–78 years) underlying OA. The PCR product (518 bases) was sequenced, and the results matched the published BMP-6 sequence reported by Celeste et al (16), with only 2 exceptions (C instead of T at position 1241 and G instead of C at position 1283, both noncoding). PCR products of BMP-6 cDNA were detected in all samples tested (Figure 1), providing

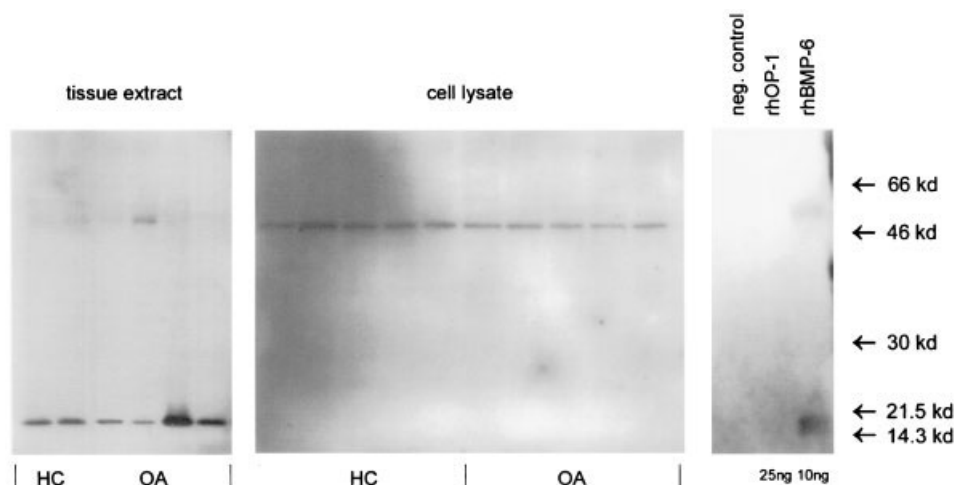


Figure 2. Detection of bone morphogenetic protein 6 (BMP-6) in cartilage extracts and cell lysates from healthy control (HC) and osteoarthritic (OA) joints. Cartilage samples from 4 OA and 2 normal joints were used for the cartilage extract studies, and 5 OA and 5 normal samples were used for the cell lysate studies. A human BMP-6-specific goat IgG antibody was used to detect the indicated growth factor in cell lysates of adult human articular cartilage under reducing conditions. Recombinant human BMP-6 (rhBMP-6; 10 ng) and recombinant human osteogenic protein 1 (rhOP-1; 25 ng) were run in parallel as controls. Sample buffer served as the negative control. Molecular weight markers are shown at the right.

evidence that BMP-6 mRNA was expressed in human articular cartilage independently of age or the presence of OA. When the densities of the BMP-6 mRNA bands

were normalized against the densities of the β -actin bands, no significant difference between healthy and OA chondrocytes could be detected.

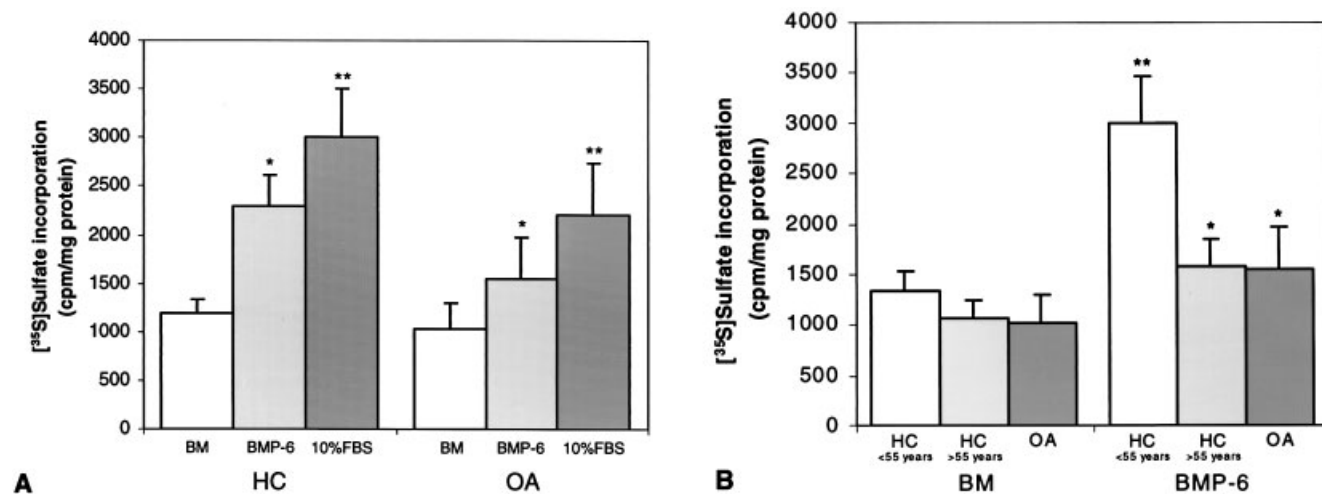


Figure 3. Bone morphogenetic protein 6 (BMP-6) stimulation of proteoglycan (PG) synthesis in healthy control (HC) and osteoarthritic (OA) human articular chondrocytes. Chondrocytes derived from 16 healthy and 12 OA cartilage samples were incubated in serum-free basal medium (BM) alone, or in BM containing BMP-6 (final concentration 100 ng/ml). On day 7 of the stimulation period, cultures were labeled with ^{35}S -sulfate for 6 hours. The radiolabel incorporated into newly synthesized matrix PGs present in the cell layer was then measured, normalized to the protein content, and expressed as cpm/mg of protein. Values are the mean and SEM. **A**, Unstimulated controls (BM), cultures stimulated with recombinant human BMP-6, and positive controls incubated with 10% fetal bovine serum (FBS). * = $P < 0.03$ versus BM; ** = $P < 0.002$ versus BM. **B**, Cartilage from healthy subjects younger than 55 years, healthy subjects older than 55 years, and OA patients. * = $P < 0.03$ versus BM; ** = $P < 0.001$ versus BM.

Expression of the active form of BMP-6 in human articular cartilage. To confirm the above results and to determine the presence of BMP-6 on the protein level, cell lysates and cartilage extracts derived from healthy and OA joints were analyzed by Western immunoblotting. We used an antibody capable of detecting both the precursor (46 kd) and the mature segment (18–23 kd) of BMP-6. In tissue extracts, bands between 14.3 kd and 21.5 kd, representing the active protein, were detected, whereas only faint signals were seen at ~46 kd. In contrast, only the pro form, at 46 kd, was detectable in the cell lysates (Figure 2).

BMP-6 stimulation of PG synthesis in healthy and OA human articular cartilage chondrocytes. To determine the stimulatory effects of BMP-6 on matrix PG synthesis, we measured the rate of ^{35}S -sulfate incorporation into chondrocytes derived from 16 healthy and 12 OA cartilage samples. Optimum concentrations of 100 ng/ml of BMP-6 were used in all experiments, as determined by dose-response curves (data not shown).

In chondrocytes from healthy cartilage, total PG synthesis was significantly increased by BMP-6 stimulation (mean \pm SEM $191 \pm 11\%$ of that in BM controls; $P < 0.001$). This increase was comparable to that in positive controls using 10% FBS ($250 \pm 17\%$ of that in BM controls; $P < 0.002$ versus BM) (Figure 3A). Interestingly, we found an age-dependent loss of response to BMP-6 among chondrocytes from healthy subjects ($r = -0.6$, $P = 0.016$) (solid regression line in Figure 4). We divided these 16 healthy controls into 2 age groups (8 subjects <55 years old and 8 subjects >55 years old), and analyzed the stimulatory effects of BMP-6 in both subgroups as compared with unstimulated controls. The results obtained in chondrocyte cultures from those <55 years old showed a significant increase in ^{35}S -sulfate incorporation into newly synthesized matrix macromolecules in the presence of BMP-6 ($224 \pm 16\%$ of that in BM controls; $P < 0.001$) after 7 days in culture (Figure 3B). Direct comparison with the group >55 years old revealed reduced PG synthesis in the older group ($P < 0.04$). Nevertheless, stimulation with BMP-6 resulted in a statistically significant increase in rate of isotope uptake in the older group compared with that in medium alone ($149 \pm 17\%$; $P < 0.03$) (Figure 3B).

In addition, we determined the responsiveness of OA chondrocytes to BMP-6, and observed that BMP-6 significantly induced the incorporation of ^{35}S -sulfate after a 7-day stimulation period, at an average of $150 \pm 25\%$ of that in BM controls ($P < 0.03$ versus unstimulated controls) (Figure 3B). There was no statistically significant difference in OA chondrocytes compared

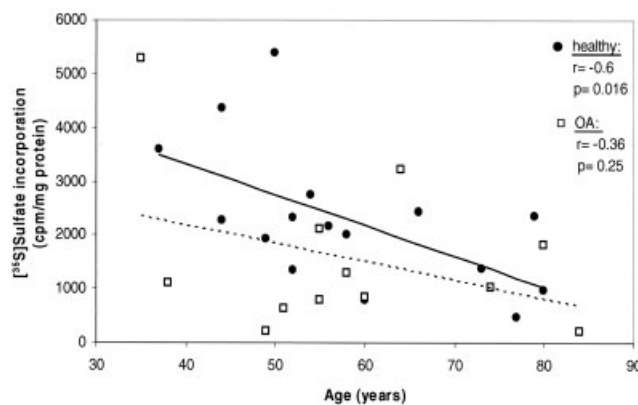


Figure 4. Decrease in responsiveness of healthy articular chondrocytes to bone morphogenetic protein 6 (BMP-6) with advancing age. Cells were cultured in serum-free basal medium without growth factors for 7 days. The rate of proteoglycan (PG) synthesis was measured by ^{35}S -sulfate incorporation into newly synthesized matrix PGs present in the cell layer. Values were normalized to the protein content and are expressed as the cpm/mg of protein. Solid regression line represents healthy specimens ($n = 16$); broken regression line represents osteoarthritic (OA) cartilage ($n = 12$).

with cultures incubated with 10% FBS ($213 \pm 24\%$ of that in BM controls; $P < 0.02$ versus BM) (Figure 3A). There was a significant difference in OA chondrocytes compared with healthy controls <55 years old ($P < 0.04$), whereas no significant difference was found in comparison with healthy controls >55 years old ($P = 0.9$). In fact, among the OA patients, there was no significant reduction in PG stimulatory capacity with age ($r = -0.36$, $P = 0.25$) (broken regression line in Figure 4), despite a mild numeric trend.

An additional analysis of chondrocyte PG biosynthesis was performed using Northern blot analysis of the cartilage marker aggrecan. Aggrecan mRNA was up-regulated after BMP-6 treatment in healthy as well as OA chondrocytes when the densities of the aggrecan mRNA bands were normalized against the densities of the β -actin bands (Figure 5).

Lack of induction of chondrocyte proliferation by BMP-6. A possible stimulatory effect of recombinant BMP-6 on cell proliferation in healthy ($n = 3$) and OA ($n = 4$) chondrocytes was determined by ^3H -thymidine incorporation. BMP-6 had no effect on the proliferation of primary human articular chondrocytes, whether from OA or normal articular cartilage samples (Figure 6). In chondrocytes incubated with 10% FBS, however, a highly significant increase in the cell proliferation rate was seen. The mean \pm SEM ^3H -thymidine incorporation in healthy samples was as follows: unstimulated samples 774.8 ± 131.6 cpm, BMP-6-stimulated samples 670 ± 78.9 , and 10% FBS-stimulated samples $3,429 \pm 551.1$

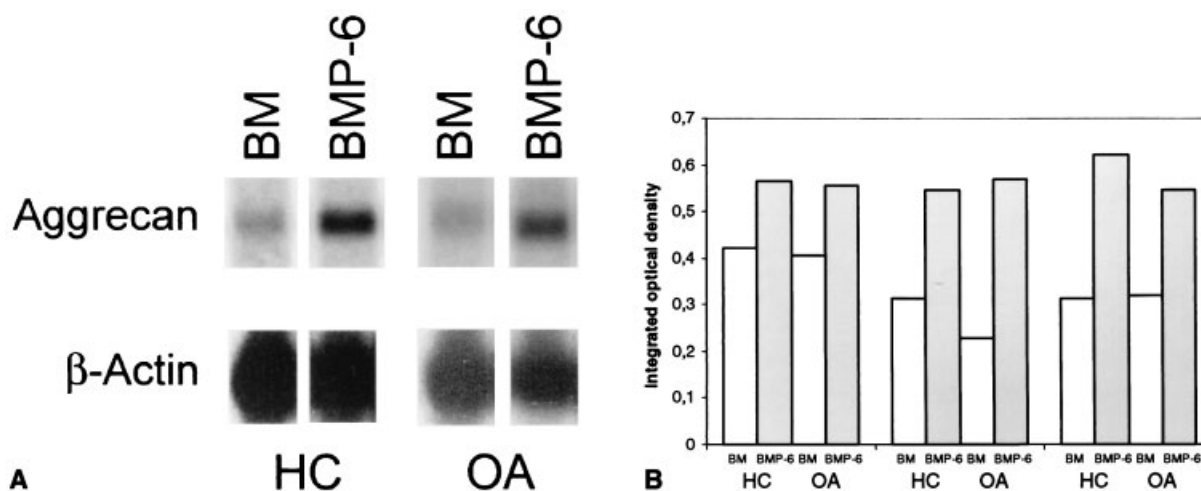


Figure 5. Northern blot analysis of the cartilage marker aggrecan and β -actin mRNA in articular chondrocytes from 3 healthy control (HC) and 3 osteoarthritis (OA) joints. Subconfluent cells were cultured in the presence or absence of bone morphogenetic protein 6 (BMP-6; 100 ng/ml) under serum-free conditions. Total RNA was isolated on day 7. Five micrograms of total RNA was used per lane and hybridized with the respective cDNA probes (see Patients and Methods). Levels of β -actin expression confirm equal loading of the mRNA. **A**, Representative blot from 3 experiments. **B**, Individual densitometry data from the Northern blots (after normalization against β -actin). BM = basal medium (unstimulated control).

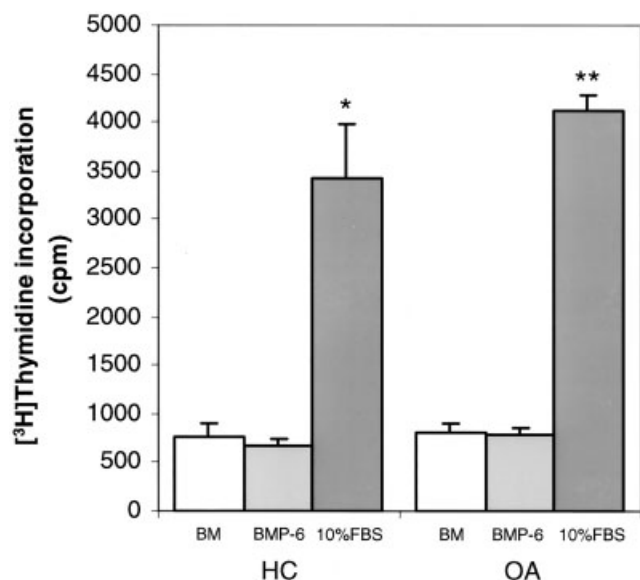


Figure 6. Lack of effect of bone morphogenetic protein 6 (BMP-6) on the proliferation of healthy control (HC) and osteoarthritic (OA) human articular chondrocytes. Serum-starved cultures of subconfluent chondrocytes derived from healthy ($n = 3$) and OA ($n = 4$) human articular cartilage samples were incubated with BMP-6 (100 ng/ml) and 10% fetal bovine serum (FBS; positive control) for 24 hours. Cultures maintained in basal medium (BM) alone served as negative controls. Cells were labeled with $1 \mu\text{Ci/ml}$ of ^3H -thymidine for 6 hours. Values are the mean and SEM. * = $P < 0.001$ versus BMP-6 and BM; ** = $P < 0.0001$ versus BMP-6 and BM.

($P < 0.001$ versus BMP-6 and BM). The mean \pm SEM ^3H -thymidine incorporation in OA samples was as follows: unstimulated samples 817.1 ± 81.5 , BMP-6-stimulated samples 787 ± 75.2 , and 10% FBS-stimulated samples $4,109.1 \pm 175.5$ ($P < 0.0001$ versus BMP-6 and BM).

DISCUSSION

The importance of members of the BMP superfamily in cartilage regeneration is well known (11,14,17–21), but the role of BMP-6 in adult human articular cartilage is currently unknown. Herein we report the presence of BMP-6 at the mRNA and protein levels in healthy as well as OA chondrocytes. Furthermore, we show that BMP-6 increased the biosynthesis of PG in stimulated chondrocyte cultures as compared with control cultures, independently of underlying OA disease. These results reveal the involvement of BMP-6 in the homeostasis of adult cartilage.

Articular chondrocytes are reported to express polypeptide growth regulators in an autocrine or paracrine manner (22–24). An increasing number of studies have implicated the presence of members of the TGF β superfamily as well as their respective receptors in normal and OA specimens (14,15,17,25–29). In the present investigation, we demonstrated the expression of

BMP-6 in adult human articular chondrocytes at the mRNA level as well as the protein level. The active and the precursor forms were found in cartilage extracts, but only the 46-kd precursor segment was found in cell lysates. A possible explanation for this difference between tissue and cultured cells may be the fact that BMP-6 is not always cleaved prior to secretion. It is estimated that in mice, 50% of the precursor protein remains intact (30). The chondrocyte isolation and culture methods used in the present study possibly affect the cleavage of the large precursor protein. However, our BMP-6 findings are paralleled by the findings by Chubinskaya and coworkers who studied OP-1. Those investigators consistently showed the presence of pro and mature forms of OP-1 in normal and OA cartilage by RT-PCR and Western blotting (15). Thus, the expression of BMP-6 found in the present study, together with the previous evidence that its respective receptors are present in healthy as well as OA chondrocytes (14), strongly suggest that BMP-6 plays an important role in the maintenance and repair of adult human articular cartilage.

Remarkably, unlike in OA cartilage, an age-related decline in the responsiveness to BMP-6 was found in healthy human articular chondrocytes. Similar age effects have been described for other molecules in studies of cartilage from animals (31–35) and humans (36), suggesting that there is a decrease in the responsiveness of articular chondrocytes to various growth factors (platelet-derived growth factors, epidermal growth factor, insulin-like growth factor 1, basic fibroblast growth factor, and TGF β) with increasing age. A possible explanation for this age-dependent effect may be a change in receptor expression. Growth factor receptors are reported to change with the differentiation status of the chondrocytes (37) as well as in culture (38). Cells obtained from older subjects may be more susceptible to such phenomena *in vitro*. Guerne and coworkers (36) showed that TGF β , the prototype growth factor of the TGF β superfamily, to which BMP-6 belongs, was the only factor among many to stimulate chondrocytes from older donors, which are often unresponsive to other factors. We therefore hypothesize that the observations of an age-related decline in responsiveness may be the result of a physiologic aging process that may predispose one to the development of disease.

Failure of successful repair of structural defects in OA cartilage is commonly believed to be caused by a disturbed equilibrium between anabolic and catabolic factors. A loss in responsiveness to growth factors may compromise tissue maintenance, leading to a disruption of the cartilage integrity. In fact, we observed a signifi-

cant loss of response to BMP-6 among OA chondrocytes compared with chondrocytes from young healthy controls (<55 years old). However, no difference could be detected between OA chondrocytes compared with healthy older chondrocytes (>55 years old). These data are reminiscent of previous reports showing a decrease in responsiveness to insulin-like growth factor 1 in OA cartilage (34,39,40). One limitation of our study design is the use of serum-free conditions, since age-related changes in different serum factors might be a confounding factor in judging growth factor responsiveness *in vivo*.

With regard to the data presented herein, the question arises whether the observed loss of response to BMP-6 plays a role in the pathogenesis of OA. On the one hand, our results suggest a reduction of stimulatory capacity in OA, when compared with that of the young healthy control group (<55 years old). On the other hand, however, the stimulatory potential of OA cells was comparable with that of articular chondrocytes derived from healthy older subjects (>55 years old) with normal cartilage, indicating a good (although not maximal) stimulatory potential of BMP-6 on total PG synthesis in OA chondrocytes. Thus, it seems unlikely that BMP-6 is involved in the pathogenesis of OA. Rather, this growth factor may be important for the maintenance of tissue homeostasis in articular cartilage.

In summary, BMP-6 is endogenously expressed in adult human articular cartilage independently of the presence of OA. Moreover, we showed that BMP-6 has the potential to stimulate PG synthesis in human articular chondrocytes derived from normal as well as OA joints. Thus, BMP-6 may be crucially involved in the maintenance/repair of human articular cartilage. Given our current dilemma in the treatment of OA, BMP-6 could therefore represent an interesting therapeutic molecule.

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