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OSTEOARTHRITIS and CARTILAGE

# Differential effects of local application of BMP-2 or TGF-β1 on both articular cartilage composition and osteophyte formation

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## Summary

Objective: The related molecules bone morphogenetic protein-2 (BMP-2) and transforming growth factor beta-1 (TGF-β1) have both been shown to stimulate chondrocyte proteoglycan (PG) synthesis *in vitro*. We investigated the *in-vivo* effects of these factors on articular cartilage PG metabolism.

Design: Several doses of BMP-2 or TGF-β1 were injected into the murine knee joint, once or repeatedly. Patellar cartilage PG synthesis was measured by [$^{35}$S]-sulfate incorporation and reverse transcriptase polymerase chain reaction (RT-PCR). PG content was analyzed by measuring safranin O staining intensity on histologic sections.

Results: A single injection of 200 ng BMP-2 induced a much earlier and more impressive stimulation of articular cartilage PG synthesis, than 200 ng TGF-β1. RT-PCR revealed that both factors upregulated mRNA of aggrecan more than that of biglycan and decorin. However, 21 days after a single injection of 200 ng TGF-β1 PG synthesis still was significantly increased, while stimulation by BMP-2 only lasted for 3 to 4 days. Stimulation by BMP-2 could be prolonged to at least 2 weeks by triple injections of 200 ng each, at alternate days. Remarkably, even after this intense exposure to BMP-2, stimulation of PG synthesis was not reflected in long-lasting enhancement of PG content of articular cartilage. In contrast, even a single injection with 200 ng of TGF-β1 induced prolonged enhancement of PG content. After repeated injections, both BMP-2 and TGF-β1 induced chondrogenesis at specific sites. 'Chondrophytes' induced by BMP-2 were found predominantly in the region where the growth plates meet the joint space, while those triggered by TGF-β1 originated from the periosteum also at sites remote from the growth plates.

Conclusions: BMP-2 and TGF-β stimulate PG synthesis and PG content with different kinetics, and these factors have different chondro-inductive properties.

Key words: BMP-2, TGF-β1, Proteoglycan, Osteophytes.

## Introduction

Loss of proteoglycans (PGs) from articular cartilage is a feature of several joint diseases. Depletion of these highly sulfated and hydrated molecules decreases the resistance of cartilage to mechanical forces and to extended enzymatic degradation and could therefore be the first step in cartilage degeneration. Factors that are able to stimulate PG synthesis and to accelerate replenishment of PGs in depleted cartilage could be of significant therapeutic value. Transforming growth factor beta-1 (TGF-β1) and bone morphogenetic protein-2 (BMP-2) have been shown to stimulate chondrocyte PG synthesis *in vitro* [1–5]. Therefore, they are possible candidates in the search for factors that improve restoration of articular cartilage. TGF-βs and BMPs belong to the TGF-β superfamily [6, 7] This superfamily consists of dimeric molecules of which each monomer contains at least seven conserved cysteine residues [8]. The proteins signal by serine/threonine kinases [6, 9]. In earlier studies [10] we showed that intra-articular injection of 200 ng TGF-β1 into the knee joint of C57Bl/6 mice induces long-lasting stimulation of patellar cartilage PG synthesis. However, we also found that multiple injection induces fibrosis (thickening of synovium and ligaments) and formation of 'chondrophytes', originating from the periosteum, and developing into osteophytes. In the present study

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we tried to identify dose regimens which might preserve the anabolic effect on cartilage without pronounced side effects on synovial tissue and periosteal tissue. Moreover, we made a comparison with BMP-2. The latter factor belongs to the BMP family and has been shown to possess strong chondrocyte PG synthesis stimulatory activity, *in vitro* [4, 5], whereas information on *in-vivo* effects on cartilage metabolism is lacking. It was found that both factors stimulate chondrocyte PG synthesis, but with markedly different kinetic profiles. Moreover, both factors induced chondrocytes, but at different, characteristic, regions of the joint.

## Materials and Methods

### ANIMALS

Male C57Bl/6 mice aged 12 weeks were used. They were fed a standard diet and tap water adlibitum.

### GROWTH FACTORS

Recombinant human BMP-2, rhTGF-β1, and rhTGF-β2 were kindly provided by Genetics Institute Inc (Cambridge, MA, U.S.A.), Genentech Inc (San Francisco, CA, U.S.A.), and Novartis (Basel, Switzerland), respectively.

### INTRA-ARTICULAR INJECTIONS

BMP-2 and TGF-βs were dissolved in saline + 0.1% ultrapure bovine serum albumin (Sigma, St Louis, MO, USA). Six microliter volumes were injected into the joint cavity of the right knee. BMP-2 and TFG-β were administered in 2, 20, 200, or 400 ng dosi. Simultaneously, the contralateral joint received an equal volume of vehicle (saline + 0.1% bovine serum albumin). Each joint was injected once or three times at alternate days.

### HISTOLOGY

Whole knee joints were dissected and processed as previously described [11]. Semiserial frontal sections were stained by hematoxylin/eosin or safranin O/fast green for examination of cells and cartilage matrix, respectively. For autoradiographic analysis of [$^{35}$S]-sulfate incorporation [11], radiolabeled sulfate (75 μCi) was injected intraperitoneally 6 h before dissection of the knee joints. After histologic processing, 6-μm sections were prepared and mounted on gelatin-coated slides. These were dipped in $K_5$ emulsion (Ilford, Basildon, Essex, U.K.) and exposed for 3–5 weeks. After this period, the slides were developed and stained with hematoxylin and eosin.

### DETERMINATION OF PATELLAR CARTILAGE PROTEOGLYCAN SYNTHESIS

Proteoglycan synthesis was measured *ex vivo* according to the method of van den Berg *et al*. [12]. Patellae with a standard amount of surrounding tissue were dissected from the knee joints. Patellae were then pulse-labeled (2 h, at 37°C) with [$^{35}$S]-sulfate. Subsequently, they were washed, fixated in ethanol, and decalcified in formic acid. After decalcification the entire cartilage was stripped off, dissolved in lumasolve and radioactivity was measured by liquid scintillation counting. In some experiments a piece of 0.2 mm$^2$ was punched out of the patellar cartilage for study of the homogeneity (central part relative to peripheral part) of growth factor effects [13].

### DETERMINATION OF PATELLAR CARTILAGE PROTEOGLYCAN CONTENT

Articular cartilage PG content is reflected in safranin O staining intensity in histological sections. This was measured using an automated image analysis system as described before [14]. Fast green staining was neutralized by use of a green filter. Optical density was examined in the non-calcified cartilage. Measurements were corrected for chondrocyte lacunae and for background measured in PG depleted cartilage in which no red stain was visible any more.

### RNA EXTRACTION AND REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

Patellae were dissected and immediately decalcified in 3.5% EDTA for 4 h at 4°C. Following decalcification, the complete articular layer was stripped from the underlying bone. The isolated cartilage was instantly put in TRIzol reagent (Life Technologies) for RNA extraction. In control experiments it was shown that this procedure did not affect the RNA isolation or RT-PCR negatively [15]. RNA was directly extracted from cartilage, without homogenization of tissue. Cartilage of 10 patellae was pooled. Before reverse transcription, the isolated RNA was treated with DNAse 1 (Life Technologies). The reverse transcription reaction was performed with

moloney-murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies), using an oligo$(dT)_{15}$ primer (Eurogentec, Liege, Belgium). Amplification of DNA was accomplished by using Taq DNA polymerase (Life Technologies) up to a cycle number of 40. To estimate the relative mRNA levels, 5 µl samples were taken at increasing cycle numbers. The PCR products were electrophorized in 1.6% agarose gels containing ethidium bromide. The cycle number at which the product was first detected on the gel was taken as a measure for the amount of specific mRNA present in the originally isolated RNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were used as an internal control. This method was validated by van Meurs *et al*. [15]. For determination of growth factor effects at one time point the RNA extracts of at least two different experiments were analyzed by RT-PCR.

The following primers were used in the amplification reactions. GAPDH 5'-AACTCCC-TCAAGATTGTCAGCA-3' (upper), 5'-TCCACC-ACCCTGTTGCTGTA-3', resulting in a 553 bp product. Biglycan 5'-AGAAGGCCTTTAGCCC-TCCTG-3' (upper), 5'-ACTTTGCGGATACGG-TTGTC-3' (130 bp product). Aggrecan primers were used as described by Grover and Roughly [16] resulting in a 501 bp product, while decorin primers were used according to Asundi and Dreher [17] (400 bp). Primers detecting murine collagen type X had the following sequences: 5'-ATACCCTTTCTGCTGCTAATGTTCTTGACC-3' (upper), 5'-TGATATTCCTGGTGGTCCTGGCAAC-3' (lower), resulting in a 387 bp product.

### STATISTICAL ANALYSIS

Differences between experimental groups were tested using the Student's *t*-test. Differences were considered significant if $P < 0.05$.

## Results

### STIMULATION OF ARTICULAR CARTILAGE PG SYNTHESIS

Dose–response studies over a 1-week period showed that one intra-articular injection of 200 ng BMP-2 increased patella cartilage PG synthesis up to three times the normal level within 2 days [Fig. 1(a)]. Thereafter PG synthesis declined and reached nearly basal levels at day 5 after injection. The stimulation of PG synthesis could not be increased beyond the level reached with 200 ng by using a higher BMP-2 dosage (400 ng, not shown). One injection with 20 ng BMP-2 stimulated PG synthesis to 150% of basal level, but this stimulation was lost within 3 days, while 2 ng was without significant effects.

A single injection of TGF-β1 also stimulated PG synthesis but the maximum level reached was lower; only twice the normal synthesis rate [Fig. 1(b)]. Moreover, this level was reached later (after 4 days) and stimulation of PG synthesis lasted longer as compared with BMP-2 injection. Significant stimulation was found up to 21 days after injection of 200 ng TGF-β (Fig. 2). Even the 20 ng TGF- β1 dose stimulated PG synthesis for at least 7 days. Triple injections did not further increase or prolong TGF-β1- induced stimulation of PG synthesis (Fig. 3). In contrast, prolonged stimulation was found after triple injections of 200 ng BMP-2. Autoradiography showed that [$^{35}$S]-sulfate incorporation was stimulated

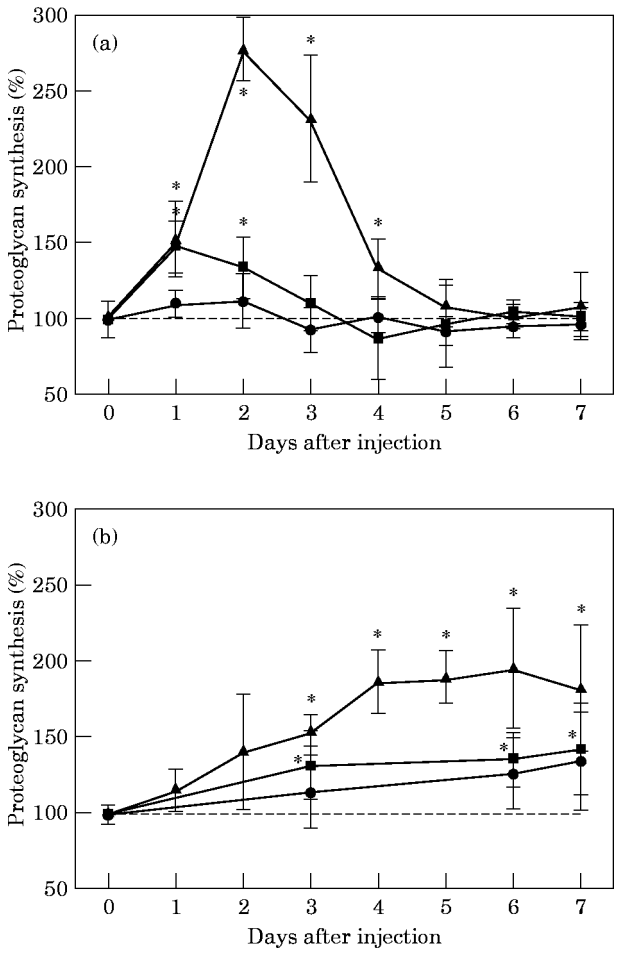



FIG. 1. Dose-dependent stimulation of patellar cartilage PG synthesis after a single injection of BMP-2 (a) or TGF-β1 (b). Even the 20 ng dose of TGF-β1 still had significant effect ($P < 0.05$) at day 7. [$^{35}$S]-sulfate incorporation is expressed as percent of the vehicle-injected control. Each value represents the mean ± S.D. of at least 12 animals. * Significant increase; ▲ = 200 ng; ■ = 20 ng; ● = 2 ng; − − − = vehicle.

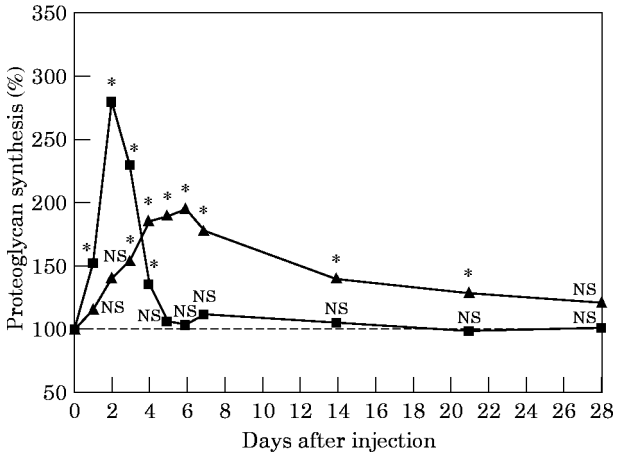



FIG. 2. Duration of stimulation of PG synthesis after a single injection of 200 ng BMP-2 or TGF-β1. The effect of BMP-2 was lost after 5 days, while TGF-β1 still had significant effect ($P < 0.05$) at day 21. [$^{35}$S]-sulfate incorporation is expressed as percent of the vehicle-injected control. Each value represents the mean of at least 12 animals. At days 14, 21, and 28 the S.D. was less than 10%. * Significant increase; NS, not significant; ■ = BMP-2; ▲ = TGF-β.

homogeneously throughout the articular cartilage both after BMP-2 and TGF-β1 injections (Fig. 4). Using scintillation counting we confirmed that effects on [$^{35}$S]-incorporation were similar in the central and peripheral parts of patellar cartilage (data not shown). Effects of TGF-β1 and TGF-β2 on proteoglycan synthesis appeared to be comparable (data not shown).

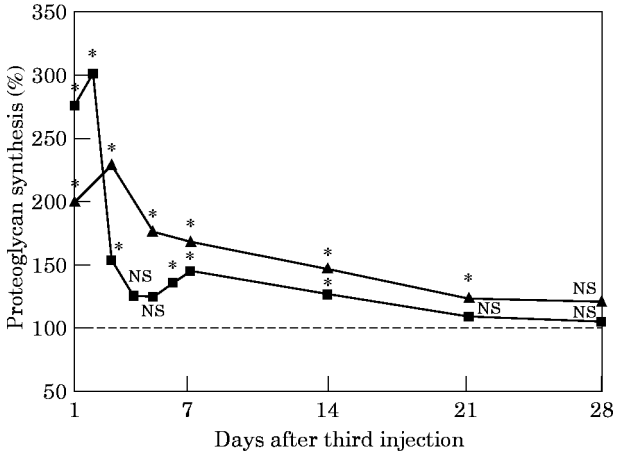



FIG. 3. Duration of stimulation of patellar cartilage PG synthesis after triple injections of 200 ng BMP-2 or TGF-β1. By giving multiple injections the effect of BMP-2 was prolonged to 14 days ($P < 0.01$), while stimulation by TGF-β1 was comparable to the effect of a single injection. [$^{35}$S]-sulfate incorporation is expressed as percent of the vehicle-injected control. Each value represents the mean of at least 12 animals. At days 14, 21 and 28 the S.C. was less than 10%. * Significant increase; NS, not significant; ■ = BMP-2; ▲ = TGF-β.

### UPREGULATION OF MRNA OF DIFFERENT PG TYPES AND OF COLLAGEN X

Overall PG synthesis can be determined by measurement of [$^{35}$S]-sulfate incorporation, but this reflects mainly aggrecan synthesis. To get insight in potential effects of BMP-2 and TGF-β1 on mRNA expression of different PG types, we used RT-PCR. Messenger RNA levels were determined of three abundant PG types in articular cartilage; aggrecan, biglycan and decorin. Results of representative RT-PCR experiments showing growth factor effects on mRNA levels of GAPDH, aggrecan, and collagen type X are presented in Fig. 5 (effects on biglycan and decorin are left out). At the time point of highest PG synthesis after the last of three BMP-2 injections, day 2, the mRNA levels of aggrecan, biglycan, and decorin were increased to eight, four and two times basal values, respectively. A single injection of 200 ng BMP-2 had less, but still significant effect (not shown); at day 2 after one BMP-2 injection, the amount of mRNA of decorin was unchanged, while that of aggrecan and biglycan had increased to four times basal values. In contrast, TGF-β injections induced upregulation of only aggrecan mRNA (two-to-four-fold) 1 day after the last of three 200 ng injections (not shown), and this effect was lost already 1 day later (Fig. 5). Interestingly, the amount of mRNA encoding for collagen type X, indicative for chondrocyte hypertrophy, was unchanged after TGF-β exposure, but highly upregulated after BMP-2 injections (±16 times normal values at day 2 after third injection).

### ELEVATION OF ARTICULAR CARTILAGE PG CONTENT

In order to elucidate whether the stimulation of PG synthesis was reflected in an increase in articular cartilage PG content, intensity of safranin O staining in histological sections was measured. This method, in contrast to biochemical analysis of total cartilage, offered the opportunity to measure especially in the uncalcified, and most reactive, cartilage layer. BMP-2 injections (3×200 ng) appeared to induce only a small, short-lived increase of patellar cartilage PG content (Table I), while TGF-β1 injections (3×200 ng) induced prolonged enhancement of the PG content, lasting at least 2 weeks after the third injection. A single injection of 200 ng TGF-β1 was almost as effective in this respect as triple injections. Effects of TGF-β1 and TGF-β2 on PG content appeared to be comparable (data not shown).

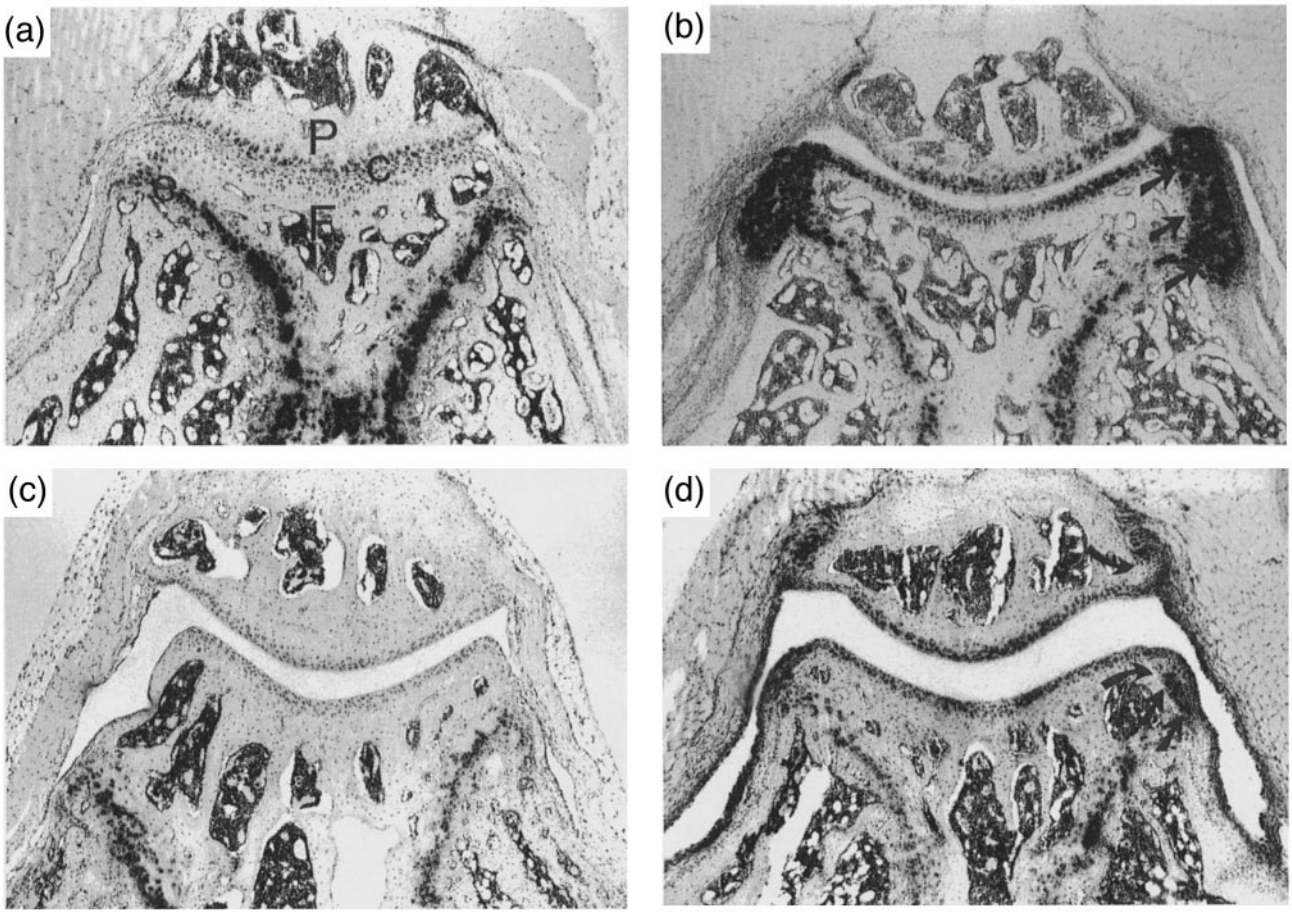



FIG. 4. Autoradiographs showing *in vivo* [$^{35}$S]-sulfate incorporation in cartilage and chondrophytes (arrows) after BMP-2 or TGF-β triple injections (200 ng each); hematoxylin/eosin stained frontal sections of murine knee joint. (a) vehicle-injected control joint of (b); (b) contralateral joint of the same animal as (a) at day 4 after the last BMP-2 injection; (c) vehicle-injected control joint of (d); (d) contralateral joint of the same animal as (c) at day 4 after the last TGF-β injection. P, patella; f, femur; c, articular cartilage; e, epiphyseal cartilage (original magnification ×100).

### Induction of osteophytes

Histologic sections of knee joints demonstrated that intra-articular injection of TGF-β and BMP-2 resulted in formation of new chondroid tissues (Figs 6 and 7). Interestingly, these new structures show different characteristics. BMP-2 induced outgrowth of epiphyseal cartilage, especially in the femur, at the level where the growth plate approaches the articular cartilage of the patellar groove. This chondrogenic activity appeared to be restricted to those regions in the growth plate adjacent to the joint space, in close contact with the periosteum. Autoradiography (Fig. 4) showed no effect of BMP-2 on $^{35}$S-sulfate incorporation in the epiphyseal cartilage, indicating local activation of only the area where the growth plate meets the joint space. Besides in the described regions, BMP-2 did not induce notable development of chondroid tissue. In contrast, TGF-β induced chondrogenesis originating from the periosteum with apparently no need for direct contact with epiphyseal cartilage. The TGF-β-induced chondroid tissues developed close to the margins of articular cartilage, at the insertion sites of ligaments, and at the base of menisci. They were found also on the patella, which has no growth plate in mice, and in superficial frontal sections of the knee, which did not include epiphyseal cartilage (Fig. 6). Chondro-inductive effects of TGF-β1 and BMP-2 together showed clear synergism of these two factors in chondrogenesis (Fig. 6). At later time points the newly formed cartilage structures lost their PGs (no safranin O staining) and developed into osteophytes, containing bone marrow (Fig. 7). For this reason the newly formed cartilage structures at early time points were termed ‘chondrophytes’. Mature osteophytes induced by BMP-2 and TGF-β looked very much alike, except for their localization. In search for treatments that stimulate articular cartilage PG synthesis for at

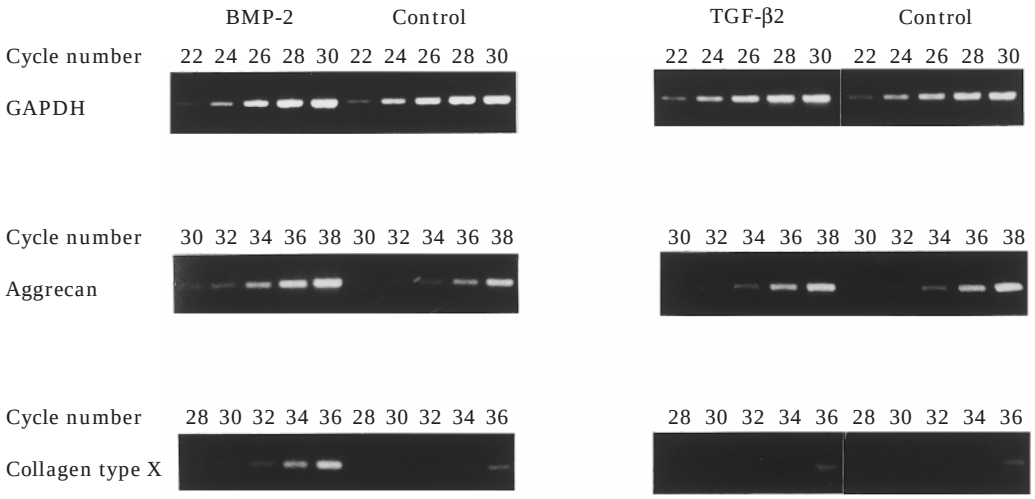



FIG. 5. Semiquantitative mRNA analysis in cartilage isolated from growth factor or vehicle injected murine joints. Cartilage was isolated 2 days after last of three injections of 200 ng BMP-2 or TGF-β2. As a control the vehicle-injected contralateral joints of the same animals were used. PCR products are shown after increasing numbers of cycles from reactions with primers specific for GAPDH, aggrecan, and collagen type X. The cycle number at which the product is first seen on the ethidium-colored gel is taken as a measure for the amount of mRNA present in the originally isolated RNA.

least a week, without inducing chondrophytes, dose–response studies were performed (Table II). These studies indicated that in the triple injection protocol chondro-induction and long-term stimulation of PG synthesis by TGF-β or BMP-2 could not be uncoupled. TGF-β was more potent than BMP-2 in both effects. The only protocol that met the above mentioned requirements, was a single injection of TGF-β. Even the 200 ng dose did not induce chondrophytes in this protocol, and as has been shown in figure 1B, a single injection of all tested TGF-β dosages (2, 20, 200 ng) induced long-term stimulation of PG synthesis.

### Induction of fibrosis

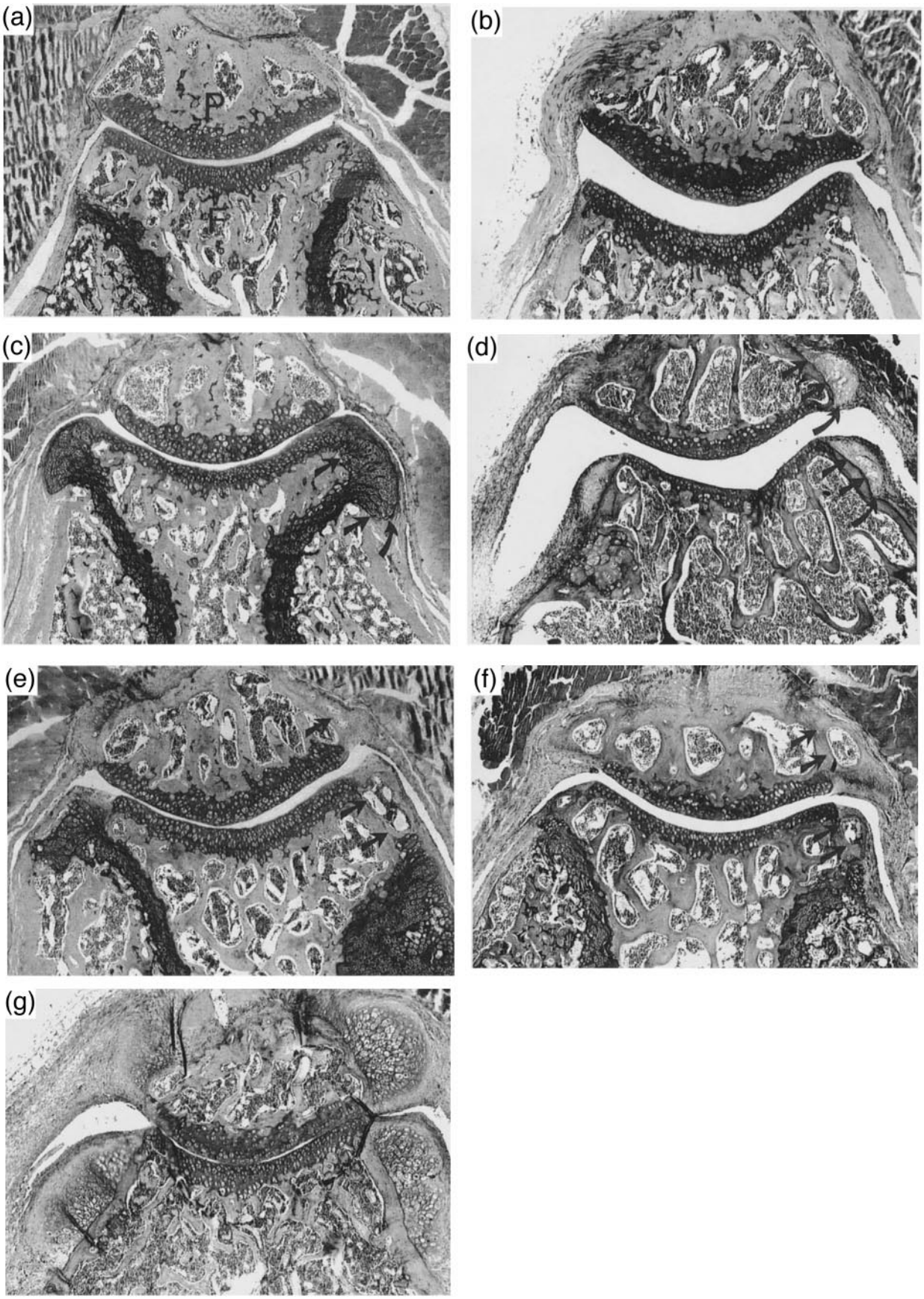
TGF-β was much more potent than BMP-2 in inducing fibrosis. Triple injections with 200 ng TGF-β increased the amount of fibroblasts in the synovial sublining, and also the diameter of collateral ligaments clearly increased (Fig. 8). BMP-2 injections had only very little effect in this respect, but in some cases a granulous tissue developed at the extraarticular side of collateral ligaments. Similar structures, but larger, were found after injections of BMP-2 + TGF-β1.

## Discussion

*In vitro*, members of the BMP family like BMP-2, BMP-4, BMP-7, and BMP-9 have been demonstrated to stimulate articular chondrocyte PG synthesis [4, 5, 18, 19], but until now no *in-vivo* data were available. We reported earlier that TGF-β1 is a potent stimulator of articular cartilage PG synthesis and content *in vivo* [10]. In the present study, we focused on the comparison of effects of intra-articular injections of TGF-β1 and BMP-2 on articular cartilage PG synthesis and content *in vivo*.

BMP-2, like TGF-β1, appeared to be a potent stimulator of articular cartilage PG synthesis

FIG. 6. Safranin O/fast green stained frontal sections of murine knee joint showing chondrophyte and osteophyte development (arrows) after triple intra-articular injections of 200 ng BMP-2 or TGF-β1. (a) Normal joint, (b) superficial section, 4 days after last BMP-2 injection, showing that remote from the growth plates no signs of chondrophyte formation were visible, (c) deeper section, at the same time-point after the last BMP-2 injection, showing chondrophytes (stained red) at the sites where growth plates meet the joint space, (d) 4 days after last TGF- β1 injection, chondrophyte development more independent of growth plate (also chondrophytes on the patella) was found; also note synovial hyperplasia, (e) 21 days after last BMP-2 injection chondrophytes had developed into osteophytes, not stained with safranin O and containing bone marrow, (f) 21 days after the last TGF-β1 injection, osteophytes had developed that looked very similar compared with those induced by BMP-2, (g) synergism of TGF-β1 and BMP-2 chondroinductive (and fibrotic) actions, 4 days afer the last injection of BMP-2 + TGF-β1 (original magnification × 100).


(a)
P
F
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(e)
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Table 1
*Effect of intra-articular injections on patellar cartilage proteoglycan content*

| Injected substance | days* | Increase of proteoglycan content (%)† | |
|---|---|---|---|
| 1×TGF-β | 7 | 16 ± 7‡ | |
| 1×TGF-β | 14 | 17 ± 8‡ | |
| 3×TGF-β | 1 | 27 ± 13‡ | |
| 3×TGF-β | 4 | 25 ± 11‡ | |
| 3×TGF-β | 7 | 22 ± 8‡ | |
| 3×TGF-β | 14 | 15 ± 6‡ | |
| 3×BMP-2 | 1 | 26 ± 15‡ | |
| 3×BMP-2 | 2 | 10 ± 10 | Not significant |
| 3×BMP-2 | 4 | 9 ± 7 | Not significant |

*Days after the last injection; in the triple injection protocol three injections (200 ng each) were given, at alternate days, meaning that in this protocol day 1 after the last injection = 5 days from start.

†Safranin O staining intensity of histological sections measured using an automated image analyzer. Staining intensity of patella cartilage in vehicle injected knee joints was stated 100%. Each experimental group consisted of at least 10 mice, and of each joint at least four sections were analyzed. ‡$P < 0.01$, significantly different from vehicle-injected joints.

*in vivo*. PG synthesis was stimulated homogeneously throughout the articular cartilage. Interestingly, stimulation of PG synthesis by BMP-2 and TGF-β1 showed different kinetics. Stimulation of PG synthesis by BMP-2 was much earlier and stronger, but also of shorter duration than stimulation by TGF-β. The retarded stimulation of PG synthesis by TGF-β could indicate that the first event is production of a second mediator, or a change in chondrocyte phenotype. An explanation of the prolonged stimulation of PG synthesis seen after TGF-β injection could be TGF-β autoinduction [20, 21], but also changes in mechanical forces on articular cartilage due to TGF-β-induced fibrosis and chondrophyte development could be responsible. In contrast to the long-term disturbance of overall PG synthesis, TGF-β induced only slight, transient changes in mRNA levels of aggrecan and did not change message of the two small PGs. Thus, on the mRNA level we found no indication of changes in the balance between large and small PGs during TGF-β-induced PG overproduction. Substantial shifts in favour of the smaller PG classes were described in the literature, but this always concerned TGF-β effects on other cell types and *in vitro* [22–24]. Reported TGF-β effects on human articular chondrocyte PG gene expression *in vitro* [25, 26] are much smaller (biglycan×2, decorin×0.5, aggrecan×4). This is more in line with our findings in murine articular cartilage *in vivo*, especially if we take into account that with RT-PCR it is not easy to pick up a shift of one cycle. BMP-2 effects on PG message were larger than those of TGF-β, and resulted in a shift in favor of aggrecan. Because of the short duration of BMP-2 effects on PG synthesis, and content, we suppose this will not have too much influence on the composition of the cartilage extracellular matrix. BMP-2 also strongly stimulated collagen type X mRNA expression. As type X collagen is synthesized primarily by hypertrophic chondrocytes [27, 28], this finding indicates that BMP-2 may be involved in induction of the hypertrophic phenotype.

Stimulation of PG synthesis was reflected in increased PG content after TGF-β, but not after BMP-2 injections. This might be caused by the short duration of BMP-2 induced stimulation of PG synthesis, but also TGF-β could promote PG binding to matrix or chondrocytes by inducing HA production [29] or by stimulation of integrin expression on chondrocytes [30]. Moreover, TGF-*β* might enhance PG content by suppressing PG degradation [1, 25, 31]. However, also members of the BMP family seem to have, at least *in vitro*, the potential to inhibit PG breakdown [19, 32].

Apart from its strong effects on cartilage PG metabolism, TGF-β was also much more potent than BMP-2 in induction of fibrosis. This was seen in the synovium, but also ligaments increased in diameter after TGF-β injections. The difference in fibrogenic properties of BMP-2 and TGF-β1 we showed is in line with a study that compared BMP-7 and TGF-β1 effects on fibroblast proliferation and matrix synthesis [33]. Another periarticular change we observed was the induction of chondrophytes, which eventually developed into osteophytes. Intra-articular injections of BMP-2 resulted in the formation of new chondroid tissue, especially in the femoropatellar area. The ability of BMP-2 to induce the formation of new cartilage and bone has been demonstrated before, using the rat ectopic

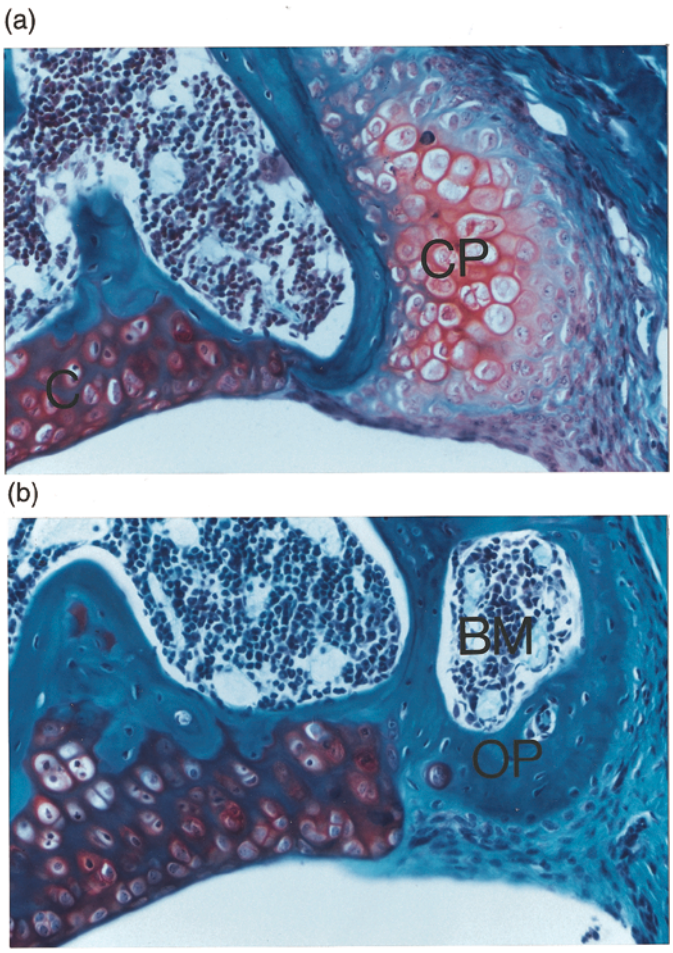


FIG. 7. Safranin O/fast green stained frontal sections of murine knee joint illustrating the transformation of TGF-β2-induced chondrophytes into osteophytes. (a) chondrophytes seen at the margins of patellar cartilage at day 5 after the last of three injections of 200 ng TGF-β2, (b) osteophytes found at the same sites one month after the last injection. CP, chondrophyte; OP, osteophyte; C, cartilage; BM, bone marrow (original magnification ×400)

bone formation assay [7, 34]. Other members of the BMP family, like BMP-3, BMP-4, BMP-5, and BMP-7 are also able to induce new cartilage and bone *in vivo* [7, 34, 35]. As we demonstrated before, intra-articular injections of TGF-β1 induced chondrophytes which develop into osteophytes [10]. Interestingly, the BMP-2-induced chondrophytes are quite different as compared with those induced by TGF-β. The BMP-2-induced chondrophytes are always growing in close contact with the area where growth plates meet the joint space, while those induced by TGF-β seem to develop more independently of these sites. Also the observation that the most pronounced TGF-β-induced chondrophyte development was on the patella (lacking a growth plate), while after BMP-2 injections chondrophytes were rarely seen on the patella, but predominantly on the femur, points in this direction. Autoradiography did not show BMP-induced activation, at the level of proteoglycan synthesis, of the growth plates themselves. This indicates that the chondroid tissue had originated from the periosteum, or from the surface of the growth plate adjacent to the joint space. Several studies performed in other systems also show that BMP-2 and TGF-β act different in inducing cartilage and bone production [36–38]. This could indicate that BMP-2 and TGF-β may act by different mechanisms, or that because of variable receptor expression they activate different cell populations. Additional evidence for the existence of differential activation pathways is the synergism in chondrophyte induction that we found after injecting BMP-2 and TGF-β into the same joint. Because of the characteristic appearance and localization of early BMP-2-induced chondrophytes, we can speculate about the relative contribution of BMP and TGF-β in osteophyte induction during natural processes in mice. From the appearance of arthritis-induced chondrophytes we conclude that there is no physiological role for BMP in this process. Moreover, the localization of osteophytes in naturally occurring and experimental osteoarthritis in mice also is more similar to that induced by TGF-β than BMP-2 injections, but we do not know what early chondrophytes in these conditions look like.

Table 2
*Correlation between long-term stimulation of PG synthesis and induction of chondrophytes by BMP-2 and TGF-β*

| Injected substance | Chondrophytes | Stimulation of PG synthesis ( > 1 week) |
|---|---|---|
| 1×200 ng TGF-β | – | + |
| 3×200 ng TGF-β | + | + |
| 3×20 ng TGF-β | + | + |
| 3×2 ng TGF-β | – | – |
| 1×200 ng BMP-2 | – | – |
| 3×200 ng BMP-2 | + | + |
| 3×20 ng BMP-2 | – | – |

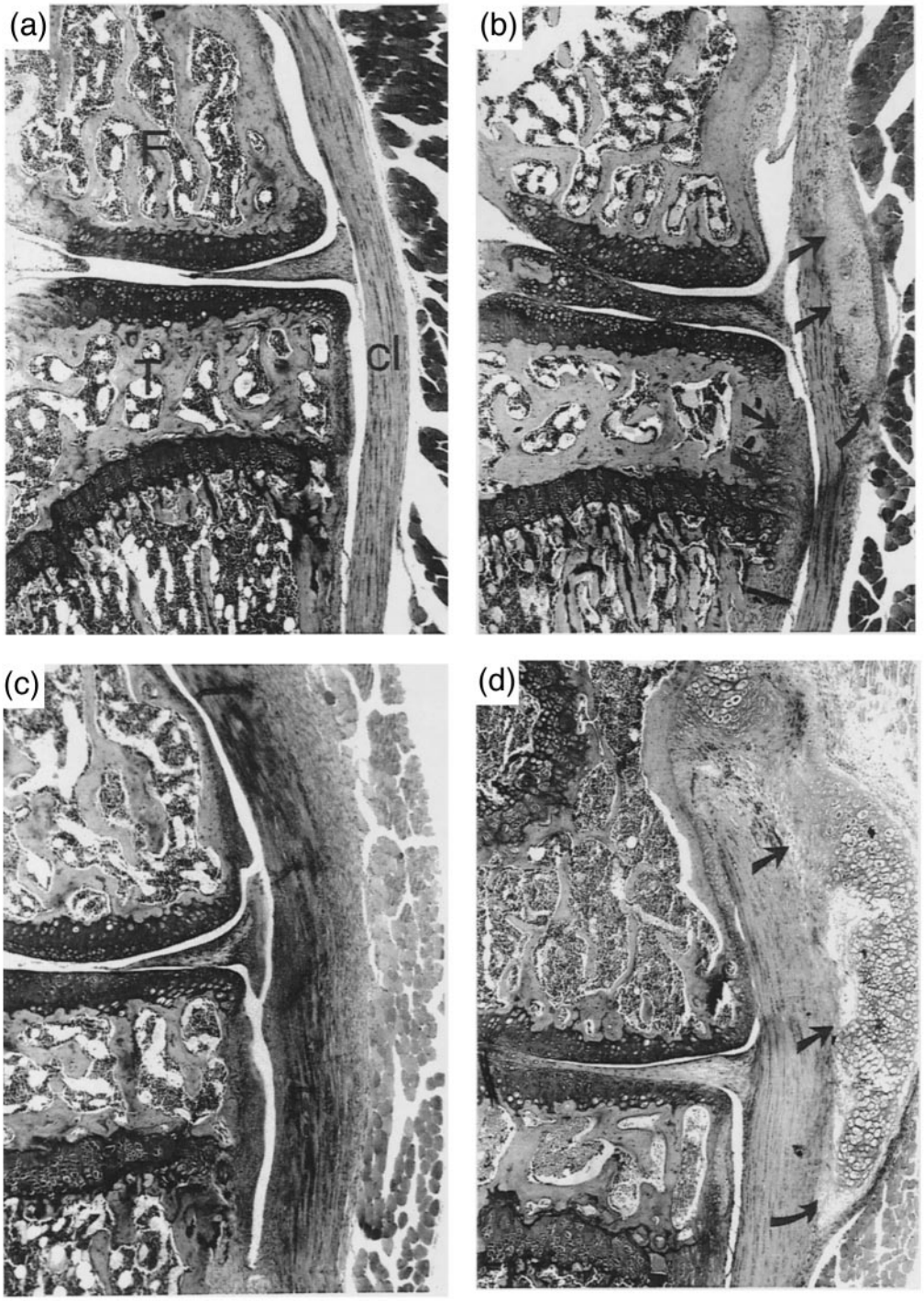


FIG. 8. Safranin O/fast green stained frontal sections of murine knee joint showing effects on collateral ligaments induced by triple intra-articular injections of 200 ng BMP-2 or TGF-β1. (a) Normal joint; (b) 4 days after the last BMP-2 injection there is no fibrosis of ligaments, but in some cases a new granulous structure, hardly stained, has developed at the extra-articular side of the ligament, and a chondrophyte is present at the end of the growth plate (arrows); (c) 4 days after the last TGF-β1 injection the collateral ligaments are thicker and in some cases they stain red instead of green, indicating enhanced PG content, presumably a sign of chondrogenesis; (d) 4 days after the last coinjection of TGF-β1 and BMP-2, with large chondrophyte at the extra-articular side of the ligament (arrows). F, femur; T, tibia CL, collateral ligament (original magnification ×100).

A single injection of 200 ng TGF-β appeared to induce long-term enhancement of articular cartilage PG synthesis and content, with only moderate fibrosis and no chondrophytes. For induction of chondrophyte formation repeated injections were needed. Three injections of a 10

times lower dose still induced chondrophyte formation. BMP-2 was less potent also in this respect, because three injections of 20 ng BMP-2 appeared to have no chondroinductive capacity. In the triple injection protocol we did not succeed in finding dosages that had significant effects on cartilage PG metabolism, without inducing chondrophytes. There is evidence that periosteum of rodents is more responsive to chondrophyte-inducing factors as compared with primates [39, 40], indicating that BMP- or TGF-β-induced formation of chondrophytes and osteophytes might be less of a problem in humans.

In summary, this study demonstrates that local administration of BMP-2 or TGF-β into normal joints stimulates articular cartilage PG synthesis. TGF-β has the highest impact, because of its long-lasting enhancement of both PG synthesis and PG content, and seems to be the most promising factor for replenishment of PG in depleted cartilage in pathological conditions. However, formation of chondrophytes might limit the therapeutic applications of TGF-β and BMP-2.

## References


1. Chandrasekhar S, Harvey AK. Transforming growth factor-β is a potent inhibitor of IL-1 induced protease activity and cartilage proteoglycan degradation. Biochem Biophys Res Commun 1988;157:1352–1359.
2. Morales TI. Transforming Growth Factor-β1 stimulates synthesis of proteoglycan aggregates in calf articular cartilage organ cultures. Arch Biochem Biophys 1991;286:99–106.
3. van Beuningen HM, van der Kraan PM, Arntz OJ, van den Berg WB. Protection fom interleukin 1 induced destruction of articular cartilage by transforming growth factor-β: studies in anatomically intact cartilage in vitro and in vivo. Ann Rheum Dis 1993;52:185–191.
4. Morris E. Differential effects of TGF-β superfamily members on articular cartilage metabolism: stimulation by rhBMP-9 and rhBMP-2 and inhibition by TGF-β. Trans Orthop Res Soc 1996;42:175.
5. Sailor LZ, Hewick RM, Morris EA. Recombinant human bone morphogenetic protein-2 maintains the articular chondrocyte phenotype in long-term culture. J Orthop Res 1996;14:937–945.
6. Reddi AH. Cartilage morphogenesis: Role of bone and cartilage morphogenetic proteins, homeobox genes and extracellular matrix. Matrix Biology 1995;14:599–606.
7. Wozney JM. Bone morphogenetic proteins. Prog Growth Factor Res 1989;1:267–280.
8. Kingsley DM. The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms. Gene Develop 1994;8:133–146.
9. Lin HY, Moustakas A. TGF-β receptors: structure and function. Cell Mol Biol 1996;40:337–349.
10. van Beuningen HM, van der Kraan PM, Arntz OJ, van den Berg WB. Transforming growth factor-β1 stimulates articular chondrocyte proteoglycan synthesis and induces osteophyte formation in the murine knee joint. Lab Invest 1994;71:279–290.
11. van den Berg WB, Kruijsen MWM, van de Putte LBA, van Beusekom HJ, van der Sluis-van der Pol M, Zwarts WA. Antigen-induced and zymosan-induced arthritis in mice: studies on in vivo cartilage proteoglycan synthesis and chondrocyte death. B J Exp Pathol 1981;61: 308–316.
12. van den Berg WB, Kruijsen MWM, van de Putte LBA. The mouse patella assay: an easy method of quantitating articular cartilage chondrocyte function in vivo and in vitro. Rheumatol Int 1982;1:165–169.
13. van Osch GJVM, van der Kraan PM, van den Berg WB. In vivo quantificatiom of proteoglycan synthesis in articular cartilage of different topographical areas in the murine knee joint. J Orthop Res 1993;11:492–499.
14. van der Kraan PM, de Lange J, Vitters EL, van Beuningen HM, van Osch GJVM, van Lent PLEM, van den Berg WB. Analysis of changes in proteoglycan content in murine articular cartilage using image analysis. Osteoarthritis Cartilage 1994;2:207–214.
15. van Meurs JBJ, van Lent PLEM, Joosten LAB, van der Kraan PM, van den Berg WB. Quantification of mRNA levels in joint capsule and articular cartilage of the murine knee joint by RT-PCR: kinetics of stromelysin and Il-1 mRNA levels during arthritis. Rheumatol Int 1997;16:197–205.
16. Grover J, Roughly PJ. Versican gene expression in human articular cartilage and comparison of mRNA splicing with aggrecan. Biochem J 1993;291:361–367.
17. Asundi VK, Dreher KL. Molecular characterization of vascular smooth muscle decorin: Deduced core protein structure and regulation of gene expression. Eur J Cell Biol 1992;59:314–321.
18. Flechtenmacher J, Huch K, Thonar EJ-MA, Mollenhauer JA, Davies SR, Schmid TM, et al. Recombinant human osteogenic protein 1 is a potent stimulator of the synthesis of cartilage proteoglycans and collagens by human articular chondrocytes. Arthritis Rheum 1996;39:1896–1904.
19. Luyten FP, Yu YM, Yanagishita M, Vukicevic S, Hammonds RG, Reddi AH. Natural bovine osteogenin and recombinant human bone morphogenetic protein-2B are equipotent in the maintenance of proteoglycans in bovine articular cartilage explant cultures. J Biol Chem 1992;267:3691–3695.
20. McCartney-Francis N, Mizel D, Wong H, Wahl L, Wahl S. TGF-β regulates production of growth factors and TGF-β by human peripheral blood monocytes. Growth Factors 1990;4:27–35.
21. Villiger PM, Lotz M. Differential expression of TGF-β isoforms by human articular chondrocytes in response to growth factors. J Cell Physiol 1992;151:318–325.
22. Border WA, Okuda S, Languino LR, Ruoslahti E. Transforming growth factor-β regulates production of proteoglycans by mesangial cells. Kidney Int 1990;37:689–695.

23. Kähäri VN, Larjava J, Uitto J. Differential regulation of extracellular matrix proteoglycan (PG) gene expression. J Biol Chem 1991;266: 10608–10615.
24. Romaris M, Heredia A, Molist A, Bassols A. Differential effect of transforming growth factor β on proteoglycan synthesis in human embryonic lung fibroblasts. Biochem Biophys Acta 1991;1093:229–233.
25. Lum Z-p, Hakala BE, Mort JS, Recklies AD. Modulation of the catabolic effects of interleukin-1β on human articular chondrocytes by transforming growth factor-β. J Cell Physiol 1996;166:351–359.
26. Roughley PJ, Melching LI, Recklies AD. Changes in the expression of decorin and biglycan in human articular cartilage with age and regulation by TGF-β. Matrix Biology 1994;14:51–59.
27. Remington MC, Bashey RI, Brighton CT, Jimenez SA. Biosynthesis of disulfide bonded short chain collagen by calf growth plate cartilage. Biochem J 1984;224:227–233.
28. Schmid IM, Linsenmayer TF. Immunohistochemical localization of short chain cartilage collagen (type-X) in avian tissues. J Cell Biol 1985;100: 598–605.
29. Ogawa Y, Sawamura SJ, Ksander A, Armstrong RM, Pratt BM, McPherson JM. Transforming growth factors β1 and β2 induce synthesis and accumulation of hyaluronate and chondroitin sulfate in vivo. Growth Factors 1990;3:53–62.
30. Loeser RF. Growth factor regulation by chondrocyte integrins. Differential effects of insulin-like growth factor 1 and transforming growth factor β on α1β1 integrin expression and chondrocyte adhesion to type VI collagen. Arthritis Rheum 1997;40:270–276.
31. Günther M, Haubeck HD, van de Leur E, Bläser J, Bender S, Gütgemann I, et al. Transforming growth factor beta 1 regulates tissue inhibitor of metalloproteinases-1 expression in differentiated human articular chondrocytes. Arthritis Rheum 1994;37:395–405.
32. Yao J, Cole AA, Huch K, Kuettner KE. The effect of OP-1 on IL-1β induced gene expressions of matrix metalloproteinases and TIMP in human articular chondrocytes. Trans Orthop Res Soc 1996;42:305.
33. Postlethwaite AE, Raghow R, Stricklin G, Ballou L, Sampath TK. Osteogenic protein-1, a bone morphogenic protein member of the TGF-β superfamily, shares chemotactic but not fibrogenic properties with TGF-β. J Cell Physiol 1994;161:562–570.
34. Wang EA, Rosen V, D'Alessandro JS, Bauduy M, Cordes P, Harada T, et al. Recombinant human bone morphogenetic protein induces bone formation. Proc Natl Acad Sci USA 1990;87: 2220–2224.
35. Sampath TK, Maliakal JC, Hauschka PV, Jones WK, Sasak H, Tucker RF, et al. Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. J Biol Chem 1992;267: 20352–20362.
36. Aspenberg P, Jeppsson C, Wang J-S, Boström M. Transforming growth factor beta and bone morphogenetic protein 2 for bone ingrowth: a comparison using bone chambers in rats. Bone 1996;19:499–503.
37. Kawaguchi H, Kurokawa T, Hoshino Y, Kawahara H, Ogata E, Matsumoto T. Immunohistochemical demonstration of bone morphogenetic protein-2 and transforming growth factor-β in the ossification of the posterior longitudinal ligament of the cervical spine. Spine 1992;17:S33–S36.
38. Roark EF, Greer K. Transforming growth factor-β and bone morphogenetic protein-2 act by distinct mechanisms to promote chick limb cartilage differentiation in vitro. Dev Dyn 1994;200:103–116.
39. Miyamoto S, Takaoka K, Yoshikawa H, Hashimoto J. Trans-filter bone induction by bone morphogenetic protein. Trans Orthop Res Soc 1993;29:99.
40. Ripamonti U, Bosch C, van den Heever B, Duneas N, Melsen B, Ebner R. Limited chondro-osteogenesis by recombinant human transforming growth factor-β1 in calvarial defects of adult baboons (Papio ursinus). J Bone Miner Res 1996;11:938–945.