EXTENDED REPORT

Stimulatory effects of distinct members of the bone morphogenetic protein family on ligament fibroblasts

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Objective: To investigate effects of cartilage derived morphogenetic protein-1 and -2 (CDMP-1, CDMP-2), bone morphogenetic protein (BMP)-7 and BMP-6 on metabolism of ligament fibroblasts and their osteogenic or chondrogenic differentiation potential.

Methods: Ligament fibroblasts were obtained from 3 month old calves, plated as monolayers or micromass cultures, and incubated with or without CDMP-1, CDMP-2, BMP-7, and BMP-6. Expression of the indicated growth factors was assessed by RT-PCR and western immunoblotting. The presence of their respective type I and II receptors, and lineage related markers, was investigated in stimulated and unstimulated cells by RT-PCR and northern blotting. Biosynthesis of matrix proteoglycans was assessed by [³⁵S]sulphate incorporation in monolayers. Alcian blue and toluidine blue staining was done in micromass cultures.

Results: CDMP-1, CDMP-2, BMP-7, and BMP-6 were detected on mRNA and on the protein level. Type I and II receptors were endogenously expressed in unstimulated ligament fibroblasts. The growth factors significantly stimulated total proteoglycan synthesis as assessed by [³⁵S]sulphate incorporation. Toluidine blue staining showed cartilage-specific metachromasia in the growth factor treated micromass cultures. Transcription analysis of stimulated ligament fibroblasts demonstrated coexpression of chondrocyte markers but no up regulation of osteogenic markers.

Conclusion: CDMP-1, CDMP-2, BMP-7, and BMP-6 and their receptors were expressed in ligament tissue. These growth factors induced matrix synthesis in fibroblasts derived from bovine ligament. The preferential expression of cartilage markers in vitro suggests that CDMP-1, CDMP-2, BMP-7, and BMP-6 have the potential to induce differentiation towards a chondrogenic phenotype in ligament fibroblasts. Thus, fibroblasts from ligaments may serve as a source for chondrogenesis and tissue repair.

Ligaments connect one bone to another and act to constrain and guide joint motion.^{1,2} They have dynamic characteristics, because they respond to exercise or immobilisation by altering their tensile strength and can be repaired after injury.^{3,4} This behaviour suggests that the resident cells can detect mechanical force and respond by altering the composition of the extracellular matrix and have a regenerative potential. In these respects, there is evidence that ligament fibroblasts are responsive to a variety of growth factors, such as fibroblast growth factor, insulin-like growth factor, or transforming growth factor (TGF) β , which stimulate either cell proliferation⁵ or matrix synthesis.⁶

Bone morphogenetic proteins (BMPs) are highly conserved signalling molecules, which exert their biological effects through binding to a heteromeric receptor complex of type I and type II serine/threonine kinase receptors.⁷⁻¹⁰ The term *bone* morphogenetic proteins, however, may be misleading, because these proteins control many different events in axis formation, cell-cell communication as well as cell and tissue differentiation,^{11,12} and initiate the development of many organ systems, such as the urinary system.¹³ Besides their importance in extraskeletal tissue the BMP-5/6/7/8 as well as the growth and differentiation factor (GDF-5/6/7) subgroups are essential for the formation of cartilaginous tissue during embryonic limb development.¹⁴⁻¹⁷ Moreover, because these morphogenes are expressed and exert their biological functions in mature articular cartilage¹⁸⁻²² a role in tissue remodelling/repair has been proposed widely.

However, a different mechanism in BMP biology was discovered by Wolfman *et al*, who reported that GDF-5/6/7,

the murine homologues to the human cartilage derived morphogenetic proteins (CDMP-1, -2, -3) may be responsible for ectopic neotendon/ligament formation in vivo.²³ This report attracted much attention, because treatment of injuries to tendons/ligament frequently results in a limited return of function, joint instability, and increased likelihood of developing osteoarthritis.²⁴⁻²⁶ In more recent animal studies a possible role of these growth factors, in particular CDMP-1, CDMP-2, and BMP-7, in ligament/tendon repair has been proposed.²⁷⁻²⁹ The cellular and molecular mechanisms induced by BMP stimulation in these tissues, however, remain unclear.

To characterise the stimulatory effects of BMPs on tendon/ligament fibroblasts we studied the effects of four distinct members of the BMP-family, CDMP-1, CDMP-2, osteogenic protein-1 (OP-1/BMP-7), and BMP-6 on the metabolism of ligament fibroblasts and their osteogenic/chondrogenic differentiation potential.

MATERIALS AND METHODS

Cell culture

The intra-articular ligaments of metacarpophalangeal joints were obtained from 3 month old calves. Isolation of ligament fibroblasts was performed as described by Nagineni *et al*.³⁰

Abbreviations: AP, alkaline phosphatase; BM, basal medium; BMP, bone morphogenetic protein; CDMP, cartilage derived morphogenetic protein; FBS, fetal bovine serum; GDF, growth and differentiation factor; PBS, phosphate buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; TGF β , transforming growth factor β

Table 1 Primer sequences of oligonucleotides

mRNA template	Primer sequence (forward)	Primer sequence (reverse)
Liver-bone-kidney alkaline phosphatase ³⁷	5'-CTG GAC CTC GTT GAC ACC TG-3'	5'-GAC ATT CTC TCG TTC ACC GC-3'
Osteocalcin ⁶⁵	5'-TAC CAC TCC TCG CCC TAT TG-3'	5'-GAA GAG GAA AGA AGG GTC CC-3'
Aggrecan ⁶⁶	5'-CGC TAC GAC GCC ATC TGC TAC-3'	5'-GCC TGC TGT GCC TCC TCA AA-3'
Type II collagen ⁶⁶	5'-ATG ACA ATC TGG CTC CCA AC-3'	5'-GCC CTA TGT CCA CAC CGA ATT-3'
Decorin ⁶⁶	5'-CAA AAT AAC CGA AAT CAA AGA-3'	5'-CGT AAG GGA AGG AGG AAG ACC-3'
Type I collagen ³⁷	5'-CGT GGT GAC AAG GGT GAG AC-3'	5'-TAG GTG ATG TTC TGG GAG GC-3'
β -Actin	5'-TGT GAT GGT GGG AAT GGG TCA G-3'	5'-TTT GAT GTC ACG CAC GAT TTC C-3'
TGF β superfamily receptor type I (ALK-1) ⁶⁷	5'-CGA CGG AGG CAG GAG AAG CAG-3'	5'-TGA AGT CGC GGT GGG CAA TGG-3'
Activin A receptor, type I (ALK-2) ⁶⁷	5'-GAG TGA TGA TTC TTC CTG TGC-3'	5'-TTG GTG GTG ATG AGC CCT TCG-3'
Bone morphogenetic protein receptor, type IA (ALK-3) ⁶⁷	5'-AAA AGT GGC GGT GAA AGT ATT-3'	5'-AGT CTG GAG GCT GGA TTG TGG-3'
Activin A receptor, type IB (ALK-4) ⁶⁷	5'-GAG ATC GTG GGC ACC CAA GGG-3'	5'-AGC TGG GAG AGG GTC TTC TTG-3'
TGF β receptor I (ALK-5) ⁶⁷	5'-GAT GGG CTC TGC TTT GTC TCT-3'	5'-TGT CTT TAT TGT CTG CTG CTA-3'
Bone morphogenetic protein receptor, type IB (ALK-6) ⁶⁸	5'-TAC AAG CCT GCC ATA AGT GAG AAG C-3'	5'-ATC ATC GTG AAA CAA TAT CCG TCT G-3'
Bone morphogenetic protein receptor, type II (BMPR-II) ⁶⁸	5'-TCC TCT CAT CAG CCA TTT GTC CTT TC-3'	5'-AGT TAC TAC ACA TTC TTC ATA G-3'
Activin type IIA receptor (ActRIIA) ⁶⁷	5'-GCA AAA TGA ATA CGA AGT CTA-3'	5'-GCA CCC TCT AAT ACC TCT GGA-3'
Activin type IIB receptor (ActRIIB) ⁶⁷	5'-CAA CTT CTG CAA CGA GCG CTT-3'	5'-GCG CCC CCG AGC CTT GAT CTC-3'
Cartilage derived morphogenetic protein-1 (CDMP-1)	5'-ACG GGA CTT GTT CTT TAA TG-3'	5'-CTT ATA CAC CAC GTT GTT GG-3'
Cartilage derived morphogenetic protein-2 (CDMP-2)	5'-GCT GGG ACG ACT GGA TTA TC-3'	5'-TAG ACC ACA TTA TTG CCC GC-3'
Bone morphogenetic protein-7 (BMP-7/OP-1)	5'-ATG GTG GCT TTC TTC AAG GC-3'	5'-TTC AGG ATG ACG TTG GAG CT-3'
Bone morphogenetic protein-6 (BMP-6)	5'-CAG GAG CAT CAG CAC AGA GA-3'	5'-ATG TGT GCG TTG AGT GGG AA-3'

Briefly, 3 cm long pieces of the intra-articular ligaments were harvested aseptically, avoiding ligament-bone insertions, and placed in sterile medium, 1:1 mixture of Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD, USA) and Ham's F12 (Life Technologies) containing 10% (vol/vol) fetal bovine serum (FBS; PAA Laboratories, Linz, Austria) and antibiotics/antimycotics (100 U/ml penicillin G, 100 mg/ml streptomycin, and 0.25 μ g/ml amphotericin B; Life Technologies). The ligaments were minced into small pieces (2–3 mm²) and grown as explant cultures in T175 flasks (Becton Dickinson, Franklin Lakes, NJ, USA) until subconfluence. Thereafter, the fibroblasts were released from the culture flasks by 6 ml/flask of trypsin-EDTA solution (Gibco BRL, Grand Island, NY, USA). All further experiments were performed using passage 1 cells after trypsin release.

For proteoglycan synthesis, alkaline phosphatase (AP) and protein assays, fibroblasts were plated in 24 multiwell plates (Costar, Cambridge, MA, USA) as monolayer cultures in quadruplicate at a density of 1×10^5 cells/well in the culture medium described above. At 90% confluence, ligament fibroblast cultures were switched to a chemically defined serum-free basal medium (BM) as described previously.³¹ CDMP-1, CDMP-2, BMP-7, and BMP-6 were used at different concentrations (10 ng/ml, 30 ng/ml, 100 ng/ml, and 300 ng/ml). Over a period of 5 days growth factors and BM were added as needed and culture medium was replaced every second day. Cell cultures maintained in BM alone served as negative controls, cultures incubated with 10% FBS represented positive controls. The plates were maintained at 37°C in humidified air and 5% CO₂.

For total RNA preparation, 1×10^6 cells were seeded in 100 mm tissue culture dishes (Costar) and cultured in the presence of the indicated growth factors at a final concentration of 100 ng/ml for 7 days.

Micromass cultures were prepared as described previously.³² The cultures were stimulated with growth factors at a final concentration of 100 ng/ml. Cells were harvested after 10 days for Alcian blue staining and histology.

Growth factors

GDF-5 (CDMP-1), GDF-6 (CDMP-2), BMP-7, and BMP-6 were obtained from R&D Systems Inc (Minneapolis, MN, USA).

Alkaline phosphatase activity

Staining of AP was done as described elsewhere³³ and evaluated by light microscopy.

For AP activity assays, cells were sonicated in 500 μ l 0.1% Triton X-100 in distilled water and treated as described previously.³⁴ Measurement was performed using an enzyme linked immunosorbent assay (ELISA) reader (Dynatech MR 7000; Guernsey Channel Islands, UK) at 405 nm. Enzyme activity is expressed as nanomoles of *p*-nitrophenol released, normalised to the protein content of the sample. Monolayer cultures of bovine periosteal cells incubated with 10% FBS served as positive controls. Total cellular protein was determined by the Bio-Rad Protein Assay according to the instructions of the manufacturer (Bio Rad Laboratories GmbH, Munich, Germany); samples were measured at 550 nm.

Biosynthesis of matrix macromolecules

Proteoglycan synthesis rate, as determined by [³⁵S]sulphate incorporation into sulphated macromolecules, was evaluated as previously described.³⁵ Fibroblast cultures were washed twice with phosphate buffered saline (PBS) and labelled with 20 μ Ci/ml of [³⁵S]sulphate (carrier-free, Amersham, Buckinghamshire, UK) in a sulphate-free/serum-free

Figure 1 Endogenous expression of BMP-specific type I and type II receptors by bovine ligament fibroblasts. Total RNA was extracted from unstimulated ligament fibroblast monolayers cultured in serum-free BM for 5 days. cDNA, prepared as described in the "Materials and methods" section, was subjected to PCR amplification using primer sequences for ALK-1 to ALK-6, ActRIIa, ActRIIb, and BMPRII. The PCR mixture was separated on a 1.5% agarose gel, stained with SYBR green and visualised on a Fluorimager system. Equal loading of the samples was confirmed by β -actin expression. The data shown are representative of three independent experiments.

medium for 3 hours at 37°C. Cultures were extracted in guanidine-HCl buffer (4 M guanidine-HCl, 50 mM sodium acetate buffered at pH 7.2). Unincorporated isotope was removed using Sephadex G-25 (PD-10 columns; Pharmacia Biotech, Piscataway, NJ, USA) gel filtration chromatography. Aliquots from void volume fractions were measured by liquid scintillation counting (1410 liquid scintillation counter; Wallac Oy, Turku, Finland) and normalised for DNA content. DNA content was assessed by the use of bisbenzimidazole (Hoechst 33258; Sigma, St. Louis, MO).³⁶ As a negative control, human foreskin fibroblasts (kindly provided by Dr Popow, Department of Virology, Medical University of Vienna) were cultured and treated identically.

RNA isolation, northern blot analysis, and RT-PCR

Total RNA was extracted using a commercially available kit (RNeasy Kit, Quiagen, Valencia, CA, USA). Northern blot analysis was performed according to standard protocols described elsewhere.¹⁸ For reverse transcriptase-polymerase chain reaction (RT-PCR) 1 µg total RNA of each sample was copied into cDNA in a 20 µl reaction using the First-Strand cDNA Synthesis Kit (Pharmacia, Uppsala, Sweden). Aliquots (1 µl) were amplified in a previously described reaction mixture.¹⁸ Amplification reactions were performed in an air thermal cycler (Idaho Technology Inc, Idaho Falls, ID, USA). The amplified DNA fragments were visualised with a Fluorimager (Biorad Laboratories, Hercules, CA, USA) and band densities were calculated using Quantity One software (Biorad). Positive and negative controls were run in parallel. Table 1 lists the primer sequences used.

Western blot analysis

Freshly isolated intra-articular ligaments of metacarpophalangeal joints from five different calves were extracted in guanidine-HCl buffer (1 M guanidine-HCl, 50 mM sodium acetate buffered at pH 7.2) in the presence of protease inhibitors for 3 hours at 4°C and subsequently dialysed (Slide-A-Lyzer Dialysis Cassette; Pierce; Rockford, IL, USA) against water for 48 hours at 4°C. Western blot analysis was performed under reduced conditions according to standard protocols described elsewhere.¹⁸ Polyclonal antibodies against CDMP-1, CDMP-2, BMP-7, and BMP-6 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) were used at a dilution of 1:100 for western blot experiments. Recombinant CDMP-1, CDMP-2, BMP-7, and BMP-6 were used as positive controls; sample buffer served as negative control. Blots were visualised on Kodak X-Omat Blue XB-1 imaging film.

Alcian blue staining

Micromass cultures were washed twice with PBS and fixed in methanol for 30 minutes at -20°C. Thereafter, cultures were rinsed with distilled water and covered with Alcian blue at pH 0.2 (0.5% Alcian blue 8 GS, (Carl Roth, Karlsruhe, Germany) in 1 N HCl). Cultures were stained overnight and washed extensively with distilled water. To quantify the intensity of the Alcian blue stain, the dye was extracted with 200 µl of 6 M guanidine HCl in distilled water for 6 hours at room temperature. The optical density of the extract was measured at 630 nm using a spectrophotometer (Dynatech MR 7000).

Figure 2 Detection of CDMP-1, CDMP-2, BMP-7, and BMP-6 in extracts from bovine intra-articular ligaments derived from the metacarpophalangeal joints (n = 5). Polyclonal antibodies against CDMP-1, CDMP-2, BMP-7, and BMP-6 were used to detect the indicated growth factor in the tissue extracts under reduced conditions. Recombinant growth factors at different concentrations were run in parallel as controls. The upper bands represent the precursor form, while the bands on the bottom represent the active protein. Sample buffer served as negative control. Molecular weight markers are shown at the right.

Figure 3 (A) Ligament fibroblasts were grown under serum-free conditions in the presence of CDMP-1, CDMP-2, BMP-7, and BMP-6 at 10, 30, 100, and 300 ng/ml, respectively, for 5 days. AP activity was determined in quadruplicate cultures as described in "Material and methods". Data are expressed as mean (SD) and are representative of three independent experiments. Enzymatic activity is calculated as nanomoles of *p*-nitrophenol (*p*-NP) liberated per minute and milligrams of total cellular protein (nmol *p*-NP/min/mg protein). BM = basal medium = unstimulated control. * $p < 0.05$, ** $p < 0.01$ v BM. (B) RT-PCR on osteogenic markers in bovine ligament fibroblast ($n = 3$). Monolayer cultures were stimulated for 7 days with CDMP-1, CDMP-2, BMP-7, and BMP-6 at a final concentration of 100 ng/ml. Cultures maintained in BM alone served as negative controls. cDNA derived from cultured periosteum derived cells were used as positive control. RT-PCR for alkaline phosphatase (AP) and osteocalcin yielded specific fragments of these osteogenic markers. The bar graphs show the data of densitometry after normalisation to β -actin. Values are the mean (SD).

Histological and immunohistochemical analysis

After the stimulation period of 10 days, micromass cultures were peeled off the wells for histological examination. The micromass

cultures were fixed in 10% formalin, embedded in paraffin, and sectioned at 5 μ m. After deparaffination the sections were stained with toluidine blue according to standard protocols.

Figure 4 (A) CDMP-1, CDMP-2, BMP-7, and BMP-6 stimulate the synthesis of matrix macromolecules in ligament fibroblasts, as determined by [³⁵S]sulphate incorporation. This figure is representative of three independent experiments. Bovine ligament fibroblasts were incubated in serum-free BM alone (BM = unstimulated control), or in BM with the addition of CDMP-1, CDMP-2, BMP-7, and BMP-6 at 100 ng/ml. On day 5 of the stimulation period cell cultures were labelled with [³⁵S]sulphate for 3 hours. The radiolabel incorporated into newly synthesised matrix macromolecules present in the cell layer was then measured, normalised to protein content, and given as counts per minute/μg DNA (cpm/μg DNA). Values are the mean (SD). **p* < 0.01 v BM. (B) Expression of the cartilage markers aggrecan and collagen type II. Monolayer cultures of bovine ligament fibroblast (*n* = 3) were incubated with recombinant CDMP-1, CDMP-2, BMP-7, and BMP-6 (100 ng/ml) for 7 days. mRNA was obtained as described in "Materials and methods". To detect the presence of aggrecan and collagen type II RT-PCR was performed. The bar graphs represent the results of the densitometry analysis after normalisation to β-actin. Values are the mean (SD) of three experiments. **p* < 0.05, ***p* < 0.01 v BM. (C) Matrix constituents, common for connective tissue were analysed by RT-PCR in growth factor stimulated ligament fibroblasts (*n* = 3). The PCR mixture was separated on a 1.5% agarose gel, stained with SYBR green. The expression of decorin and collagen type I was visualised on a Fluorimager system. The bar graphs show the densitometry data normalised to β-actin. Values are the mean (SD).

For immunohistochemistry, sections were treated as described before³⁷ using a mouse antihuman type II collagen monoclonal antibody (Chemicon International, Hofheim, Germany) diluted 1:100 in 0.5% bovine serum albumin in PBS.

Statistical analysis

Data are expressed as mean and standard deviation (SD). Statistical analysis was performed by Kruskal-Wallis analysis of variance for dose-response curves and by Student's *t* test to

compare treated with untreated samples. Significance was defined as a p value <0.05 .

RESULTS

BMP-specific type I and II receptors are endogenously expressed in ligament fibroblasts

To exert their effects on target cells, BMPs bind to a specific heteromeric receptor complex consisting of type I and type II receptors. Here we examined the presence of BMP-specific type I and II receptors in bovine ligament fibroblasts by RT-PCR of total RNA obtained from monolayer cultures. We detected mRNAs encoding for type I (activin-receptor like kinase; ALK-1 to ALK-6) as well as type II (ActRII and BMPRII) receptors in the resident cells (fig 1).

Presence of CDMP-1, CDMP-2, BMP-7, and BMP-6 on the RNA and protein level in bovine ligament fibroblasts

To determine the presence of CDMP-1, CDMP-2, BMP-7, and BMP-6 in ligament fibroblasts, cultured as monolayers, we performed RT-PCR on total RNA. The PCR analysis of CDMP-1, CDMP-2, BMP-7, and BMP-6 yielded fragments specific for these growth factors in bovine ligament fibroblasts (data not shown).

Furthermore, to elucidate the presence of the distinct growth factors on the protein level, western immunoblot analysis on ligament tissue extracts was performed. We used specific antibodies against CDMP-1, CDMP-2, BMP-7, and BMP-6. The recombinant proteins served as positive controls. Our results show the presence of the indicated growth factors in tissue sample extracts of five different animals. Because the antibodies detected both the precursor and the mature growth factor segments, two bands were visualised at their appropriate sizes (figs 2A-D). When the densities of the bands were compared, the bands representing the precursors were much stronger than those of the mature (lower molecular weight) forms.

CDMP-1, CDMP-2, BMP-7, and BMP-6 did not induce osteogenic differentiation in ligament fibroblasts

AP activity is an early marker of osteogenic differentiation that spontaneously appears during culture, as shown for periosteum derived cells.³⁸ Because growth factors of the BMP family are well known to induce bone formation, we investigated whether CDMP-1, CDMP-2, BMP-7 or BMP-6

have the potential to induce osteogenic differentiation in ligament fibroblasts. In our culture system, only unstimulated monolayers showed rare AP positive cells after AP staining (data not shown). However, we observed no stimulation of AP activity after 5 days of stimulation with CDMP-1, CDMP-2, BMP-7 or BMP-6 compared with unstimulated controls; furthermore, the incubation with CDMP-1 significantly inhibited AP activity in ligament fibroblasts (fig 3A). After extending the culture period to 12 days, AP activity could not be seen in either stimulated or in unstimulated ligament fibroblast cultures. In contrast, in periosteum fibroblast cultures, which were run in parallel, AP activity was increased without incubation with the indicated growth factors (data not shown).

We additionally performed PCR analysis of the bone markers AP and osteocalcin. When densities of mRNA bands were normalised against β -actin, no difference between stimulated and unstimulated samples could be detected, indicating no differentiation towards the osteogenic lineage (fig 3B).

In vitro induction of chondrogenic differentiation by stimulation with CDMP-1, CDMP-2, BMP-7, and BMP-6

To assess the metabolic effects of CDMP-1, CDMP-2, BMP-7, and BMP-6 on matrix proteoglycans, we measured [³⁵S]sulphate incorporation into newly synthesised macromolecules, because the presence of highly sulphated proteoglycans reflects a unique biochemical feature of cartilage proteoglycans.³⁹ We observed a concentration dependent increase in isotope uptake in all growth factor treated samples after 5 days in culture. The optimal concentration of all used growth factors was 100 ng/ml (fig 4A). Compared with unstimulated controls CDMP-1 (100 ng/ml) led to a mean (SD) increase of 244 (3.7)% ($p<0.01$) in [³⁵S]sulphate incorporation, CDMP-2 (100 ng/ml) led to an increase in matrix proteoglycan biosynthesis of 168.7 (7.2)% ($p<0.01$), BMP-7 (100 ng/ml) an increase of 249.1 (5.7)% ($p<0.01$), and the stimulation with BMP-6 resulted in a 329.6 (13.9)% ($p<0.01$) increase of proteoglycan synthesis. In human foreskin fibroblasts, which were cultured under the same conditions with and without the addition of CDMP-1, CDMP-2, BMP-7, and BMP-6 (concentration 100 ng/ml), no increase in [³⁵S]sulphate incorporation could be detected (data not shown).

Figure 5 Alcian blue staining of micromass cultures of ligament fibroblasts. High density cultures of ligament fibroblasts were grown in quadruplicate with and without the addition of 100 ng/ml of either CDMP-1, CDMP-2, BMP-7 or BMP-6 under serum-free conditions. Micromass cultures maintained in BM alone served as negative controls. After a stimulation period of 10 days Alcian blue staining was performed. The column graphic represents the optical densities of the corresponding wells measured at 630 nm. The experiment was performed twice; the figure shows one representative experiment. * $p<0.01$ v BM; ** $p<0.001$ v BM.

Figure 6 Histological section of ligament fibroblast micromass cultures. Ligament fibroblasts were grown at a density of 2×10^7 cells/ml and stimulated with CDMP-1, CDMP-2, BMP-7 or BMP-6 (100 ng/ml) for 10 days. Thereafter, the micromass cultures were prepared for histological analysis and stained with toluidine blue according to standard protocols. Toluidine blue staining was performed in three independent experiments. (A) Unstimulated control, maintained in BM; (B) treatment with CDMP-1; (C) with CDMP-2; (D) stimulation with BMP-7; and (E) with BMP-6.

Given the stimulatory potential of CDMP-1, CDMP-2, BMP-7, and BMP-6 on proteoglycan synthesis in ligament fibroblasts, RT-PCR as well as northern blot analysis was performed to quantify the gene expression of aggrecan, the major cartilage matrix component,⁴⁰ and decorin, the most common core protein of proteoglycans found in tendons/ligaments.⁴¹ As shown in fig 4B, aggrecan was significantly up regulated in the growth factor treated samples compared with unstimulated controls, while decorin expression remained unchanged after stimulation with CDMP-1, CDMP-2, BMP-7, or BMP-6 (fig 4C). These results were verified by northern blotting (data not shown) and further support the transformation into a cartilage-like phenotype.

For collagen synthesis, type II collagen was present in both stimulated and unstimulated cultures. When we normalised the densities of the bands against those of β -actin, no striking difference could be detected, as assessed by RT-PCR (fig 4B) and northern blot (data not shown) and, additionally, in micromass cultures by immunohistochemistry (data not shown). The expression of type I collagen also remained unchanged during culture (fig 4C).

To further strengthen our data on the differentiation toward the chondrocyte phenotype, we set up a micromass culture system and investigated by Alcian blue staining the induction of chondrogenesis after a 10 day stimulation period. The stained micromass cultures consistently reflected the [35 S]sulphate incorporation results by exhibiting intense staining in growth factor treated samples and an almost negative stain in untreated cultures (fig 5).

Moreover, histochemical analysis of the micromass cultures with toluidine blue displayed a cartilage-specific metachromasia with toluidine blue dye for all growth factor treated samples, while none was seen for unstimulated controls (fig 6). Haematoxylin/eosin staining did not disclose the presence of fibrous bundles as seen in fibrocartilage (data not shown), letting us identify the tissue as hyaline-like.

DISCUSSION

The stimulatory effects of BMPs on ligament/tendon fibroblasts are poorly understood. Although it is known that the ectopic induction of ligamentous/tendinous tissue can be induced by BMPs,²³ a possible role of these growth factors in ligament/tendon repair has been proposed more recently.^{27–29} In our study we provide evidence that these particular fibroblasts have the potential to differentiate towards a chondrogenic phenotype under defined in vitro conditions.

BMPs exert their effects through complex formation with a heteromeric receptor complex, consisting of type I and type II transmembrane serine/threonine kinases.^{7–10} The presence of BMPs and their specific receptors in tendon/ligament fibroblasts is implicated by several studies on BMP induced tendon/ligament repair. However, the expression of these receptors has mainly been shown in pathological situations of ossified posterior longitudinal ligaments or around the calcified zone at the insertion of the ligamentum flavum to the bone.^{42–43} Similar expression patterns have been seen for the growth factors BMP-7, BMP-4 as well as BMP-2 and CDMP-1.^{42–45} In human patellar tendon samples expression of BMP-12 was found to be located in tendoblasts in the regions of active remodelling, suggesting that BMPs have a role in tissue regeneration in tendons.⁴⁶

Our results consistently demonstrated the presence of type I and type II BMP receptors in cultured, unstimulated ligament fibroblasts at the mRNA level. In addition, we showed the endogenous expression of four distinct members of the BMP family—namely, CDMP-1, CDMP-2, BMP-7, and BMP-6, at the mRNA level as well as at the protein level. The detection of both the precursor and the active protein, in conjunction with the presence of specific serine/threonine kinase receptors, emphasises the role of these growth factors in the homeostasis and repair of ligament and tendon tissue.

In the developing limb, fetal tendon fibroblasts are not irreversibly committed to a tendon cell fate, but also

contribute to other tissue lineages within the limb, such as periosteum or cartilage.⁴⁷ This suggested the ability to alter their phenotype as seen during embryogenesis, where tendons and ligaments appear to convert to fibrocartilage at their entheses.⁴⁸ This potential is preserved in adult tissue because tendon/ligament fibroblasts can change their phenotype by converting to fibrocartilage under compression force.^{49–51} The development of cartilage, which comprises a continuous spectrum of tissues between dense fibrous connective tissue and hyaline cartilage,⁵¹ is seen at sites where tendons and ligaments wrap around bony pulleys and where they attach to bone at their entheses.^{52–54} A hallmark of this transformation is the accumulation of the large proteoglycan, aggrecan, which gives the tissue the ability to withstand compressive load.^{54–56} Aside from TGF β , BMP-2 may also be involved in this process.^{57, 58}

Given the importance of these proteins in the formation of cartilage in tendon/ligament tissue, we investigated whether CDMP-1, CDMP-2, BMP-7, and BMP-6, growth factors well known to have a role in articular cartilage biology,^{18, 21, 22, 59} induce chondrogenic differentiation in ligament fibroblasts. We measured [³⁵S]sulphate incorporation into newly synthesised macromolecules in BMP treated ligament fibroblasts and showed a significant increase in its uptake, while no increase could be seen in human foreskin fibroblasts cultured under the same conditions. Moreover, we performed Alcian blue and toluidine blue staining, and demonstrated an increased synthesis of sulphated macromolecules. These data accompanied by an up regulation in aggrecan mRNA expression, as well as a steady state in decorin mRNA expression, provide further evidence that members of the BMP family are involved in the formation of cartilage-like tissue in tendons/ligaments. Thus, similar to TGF β ,⁵⁷ all tested BMPs increased aggrecan mRNA levels and left decorin expression unaffected.

With regard to collagen synthesis, the presence of type II collagen expression in the control ligament cultures may hint at the existence of precursor cells or fibroblasts at a different stage of differentiation that preferentially outgrow other cells in culture. The predisposition of these cells to synthesise type II collagen might be an explanation for the chondrogenic potential of ligament tissue. However, RT-PCR as well as northern blot analysis showed no appreciable increase in the mRNA levels for type II collagen under the culture conditions employed here. It has been reported previously, that the steady state mRNA levels for type II collagen in chondrocytes cultured in the presence of CDMP-1 and CDMP-2, BMP-7 or BMP-2 were unaffected.^{22, 60, 61} However, the concomitant expression of type II collagen and aggrecan seen here resembles a situation that serves as a reliable marker of a mature chondrogenic phenotype.⁶²

Our results are finally supported by the studies of Forslund and Aspenberg, who found cartilage and bone neoformation at sites where tendon tissue had been treated with CDMP-2 or BMP-7, whereas in untreated controls no such transformation could be seen.^{63, 64} These findings support the notion that the chondrogenic differentiation seen in our study may be due to a direct effect of the stimulation with CDMPs/BMPs.

In conclusion this study demonstrated the presence of four distinct members of the BMP family as well as their respective transmembrane receptors in fibroblasts derived from bovine ligament. Moreover, it characterised the stimulatory effects of CDMP-1, CDMP-2, BMP-7, and BMP-6 in ligamentous tissue on the expression of several connective tissue-specific markers. We are aware that this study did not show whether the response to BMPs is a uniform property of all cultured tendon fibroblasts, or whether the responding population is only a subset at a different differentiation stage.

However, the preferential induction of chondrogenic differentiation over osteogenesis provides evidence that, on the one hand, BMP augmented ligament/tendon repair could be confounded by the formation of cartilage tissue, and on the other hand, our data on the plasticity of ligament fibroblasts may reflect a first step towards a possible employment of these cells in the repair of joint surface defects, such as occurs after trauma or in osteoarthritis.

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