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Bone morphogenetic protein (BMP)-2 enhances the expression of type II collagen and aggrecan in chondrocytes embedded in alginate beads

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Summary

Objective: For autologous chondrocyte transplantation (ACT) chondrocytes are expanded *in vitro*. During expansion these cells may dedifferentiate. This change in phenotype is characterized by a raised expression of type I collagen and a decrease in type II collagen expression. Since high expression of type II collagen is of central importance for the properties of hyaline cartilage, we investigated if the growth factor bone morphogenetic protein-2 (BMP-2) may modulate the chondrogenic phenotype in monolayer cell cultures and in three-dimensional culture systems.

Design: Chondrocytes from articular knee cartilage of 11 individuals (average age: 39.8 years) with no history of joint disease were isolated and seeded either in monolayer cultures or embedded in alginate beads in presence or absence of human recombinant BMP-2 (hr-BMP-2). Then, cells were harvested and analysis of the chondrogenic phenotype was performed using quantitative RT-PCR, immunocytochemistry and ELISA.

Results: Addition of BMP-2 to chondrocytes expanded in two-dimensional (2D) cultures during the first subculture (P1) had no effect on mRNA amounts encoding type II collagen and interleukin-1β (IL-1β). In contrast, seeding chondrocytes in three-dimensional (3D) alginate cultures raised type II collagen expression significantly and addition of BMP-2 enhanced this effect.

Conclusions: We conclude that chondrocytes during expansion for ACT may benefit from BMP-2 activation only when seeded in an appropriate 3D culture system.

Key words: Autologous chondrocyte transplantation, Tissue engineering, Alginate cultures, BMP.

Introduction

Full thickness chondral defects in human adult articular cartilage have been a major problem for orthopaedic surgeons and is still challenging because of its poor self-healing capacity[1]. The presence of isolated injuries in an articular cartilage is a well known risk factor for more extensive joint damage, which untreated may result in early osteoarthritis (OA). To prevent this evolution, several therapeutic strategies have been developed: tissue response techniques (drilling or microfracture), osteochondral transplantation (OCT or "mosaicplasty") as well as the transplantation of periosteum and perichondrium in order to provide a resurfacing of the damaged cartilage surface[2]. In 1994, Brittberg and colleagues reported the first clinical results of transplantation of human autologous chondrocytes cultured in a monolayer system *in vitro*[3]. Autologous chondrocytes transplantation (ACT) is suitable even for large joint defects and may result in better cartilage repair, concerning clinical outcome as well as histology of the repair tissue, when compared to other techniques[4,5]. Autologous chondrocytes are prepared from a cartilage biopsy arthroscopically obtained from a less-weight bearing area of the joint and expanded *in vitro*. In a second open knee surgery, the expanded cells are injected into the cartilage defect, which was watertight covered before with a periosteal flap[3]. However, although such surgeries were applied successfully for more than a decade now, sequela of a wide arthrotomy represent the major disadvantage of this technique and therefore methods to seed the chondrocytes onto scaffolds to be fixed into the defect by arthroscopic or at least minimally invasive techniques are under investigation.

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Further, cartilage is a tissue rich in extracellular matrix with few chondrocytes interspersed. Consequently, the yield of chondrocytes which can be harvested from small biopsies is very limited. To achieve the required amount of cells needed for the defect, chondrocytes must undergo several population doublings *in vitro*, which strictly correlates with a dramatic loss of the chondrogenic phenotype. Chondrocytes grown in monolayer culture undergo a characteristic process of dedifferentiation, marked by a loss of type II collagen and aggrecan expression and the induction of type I collagen expression. Dedifferentiated chondrocytes do not re-differentiate sufficiently after several population doublings of *in vitro* culture[6]. In contrast, systems that support the rounded cell morphology may maintain the differentiated chondrogenic phenotype[7,8]. The proliferation rate of chondrocytes is decreased under these conditions when compared to monolayer cultures. For this reason, a suitable compromise has to be found between cell proliferation rates required for e.g., ACT and the degree of differentiation tolerable for tissue engineering of articular cartilage.

Therefore we tested the re-differentiation potential of chondrocyte cultures in alginate beads in presence of bone morphogenetic protein-2 (BMP-2). The BMP-2 is a member of the TGF-β superfamily and has been shown to play a role in chondrocyte differentiation and matrix maturation. BMP-2 is known to prevent dedifferentiation of chondrocytes grown in monolayer cultures[9–11]. However, the effect on the chondrogenic phenotype of human recombinant BMP-2 (hr-BMP-2) on human chondrocytes cultured in monolayer and alginate beads has been less characterized. Specifically, the effect of growth factor-supplemented cell culture media on the expression of inflammatory cytokines such as interleukin-1β (IL-1β) and IL-18 are still unclear. Therefore we applied BMP-2 to human articular chondrocytes cultured in monolayer and in alginate beads, and investigated the metabolic effects monitoring messenger RNA (mRNA) levels for marker genes required for cartilage regeneration after ACT and levels of inflammatory cytokines by quantitative polymerase chain reaction (RT-PCR).

Materials and methods

PREPARATION OF HUMAN CHONDROCYTES

Articular knee cartilage from 11 human donors (4 females and 7 males; average age: 39.8 years; min: 19; max: 77), with no history of inflammatory joint disease and without known infectious disease, was obtained from the local trauma surgery hospital according to their protocols. The study was approved by the local ethics committee (Medical Faculty of the University of Tübingen). Cartilage appeared macroscopically normal and free of osteoarthritic lesions in all biopsies included. Cells from each patient's tissue were isolated, cultured and treated separately. Cartilage samples were first washed twice in a phosphate buffer solution (PBS; BioWhittaker, Verviers, Belgium) and then minced. Extracellular matrix was digested overnight at 37°C by incubation of the tissue in 2.5 mg/ml type II collagenase (Roche, Mannheim, Germany) in Dulbecco modified Eagle medium (DMEM/Ham's F12; BioWhittaker, Verviers, Belgium) containing 5% human serum.

PROLIFERATION OF CHONDROCYTES IN PRIMARY CULTURES

Isolated chondrocytes were resuspended by pipetting several times and filtered through a 100 μm filter to remove undigested cartilage fragments and extracellular matrix remnants. After centrifugation the cells were resuspended in DMEM/F12 (2:1) cell culture medium supplemented with 10% human serum. Chondrocytes were seeded as primary cell culture in 75 cm^2 culture flasks (BD Falcon™; Heidelberg, Germany) at low density (5×10^3 cells/cm^2) and cultured without any antibiotics at 37°C in a humidified atmosphere containing 5% CO_2. At confluency the primary cell cultures were harvested using trypsin–EDTA (BioWhittaker; Verviers, Belgium) and passaged.

RE-SEEDING CELLS IN MONOLAYER

1.5×10^6 passaged cells were seeded in four 75 cm^2 culture flasks. Cells were cultured with DMEM/F12 (2:1) cell culture medium with 10% human serum. Two flasks were supplemented with 100 ng/ml hr-BMP-2, and two flasks were with medium alone. After 2 weeks cells were harvested for mRNA isolation and gene expression analysis. Part of the control cells (untreated) were passaged and cultured into monolayer or alginate P2 (Fig. 1).

ENCAPSULATION IN ALGINATE BEADS

The preparation of chondrocytes in alginate beads was performed as described[12], with slight modifications: chondrocytes at passage 1 (P1) were resuspended in 1.2% low viscosity alginate solution (Sigma, Taufkirchen, Germany) at a density of 5×10^5 cells/ml. Beads were formed by dispersing the alginate-cell suspension dropwise into a 102 mM $CaCl_2$ solution (Sigma, Taufkirchen, Germany) through a 22-gauge needle attached to a syringe. The beads were swirled in the $CaCl_2$ solution for 10 min to allow polymerisation and then were washed twice in PBS before immersion in DMEM/F12 (1:1) cell culture medium supplemented with 10% human serum. Chondrocytes encapsulated in alginate beads were cultured 2 weeks in medium alone, or in presence of 100 ng/ml r-BMP-2, respectively (Fig. 1).

SOLUBILIZATION OF ALGINATE BEADS

For chondrocyte recovery, culture medium was aspirated and cells were washed twice with PBS. To dissolve alginate beads, 5 mM EDTA in PBS (Roth, Karlsruhe, Germany) were added. After incubation for 15–30 min at room temperature, the solubilized alginate was removed by centrifugation at 1000 RPM for 10 min. The cell pellet was washed twice with PBS and used for RNA extraction.

GENE EXPRESSION ANALYSIS

The RNA was extracted from cells using the RNeasy Mini Kit (Qiagen Inc.; Valencia, CA) according to the manufacturer's instructions. Complementary DNA (cDNA) was obtained by reverse transcription of 1 μg mRNA (BD Clontech, Heidelberg, Germany) using oligo dT as primer. Reverse transcription was performed in a 20 μl volume in a gradient thermocycler PCR (Whatman Biometra; Göttingen, Germany). To investigate the expression of chondrocyte specific marker genes and inflammatory cytokines we used a real-time quantitative PCR (Light Cycler; Roche, Mannheim, Germany). Gene sequences were amplified using specific target primers optimized for this method (SearchLC; Heidelberg, Germany). The PCR was performed with the Light Cycler FastStart DNA SybrGreen kit (Roche, Mannheim, Germany) according to the protocol provided. The cDNA copy numbers in the respective

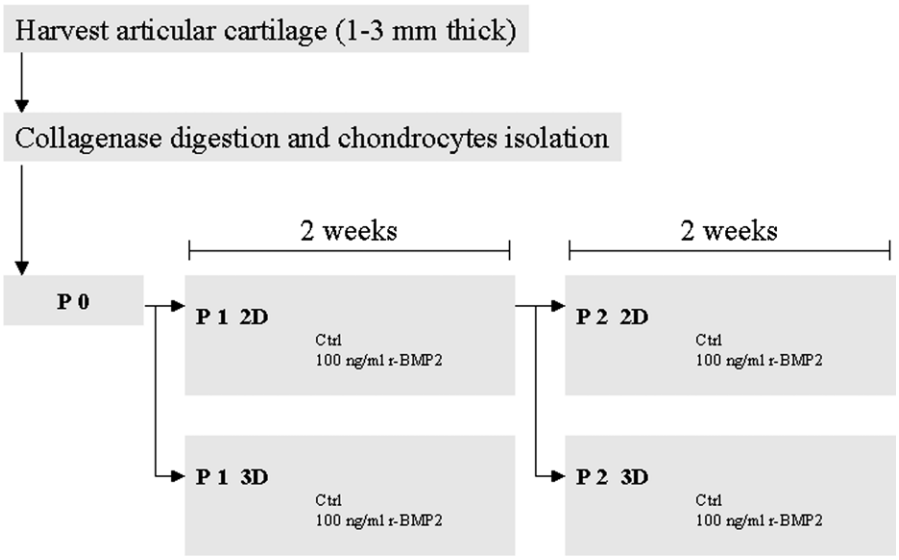

Fig. 1. Chondrocyte culture methods. After cell isolation chondrocytes were expanded in monolayer for 7–10 days. At pre-confluence cells were passaged and either cultured in monolayer (P1-2D) or encapsulated in alginate beads (P1-3D). Both culture types had medium alone or medium supplemented with 100 ng/ml hr-BMP-2. After 2 weeks cells were harvested for gene expression analysis. Part of the P1-2D chondrocytes were further passaged to establish P2 monolayer cultures as well as P2 alginate cultures with same culture conditions as at P1.

samples were calculated from a standard curve obtained by blotting known input concentrations to the PCR cycle number (CP). The copy number was normalized by the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To control the specificity of the amplification a melting curve analysis was performed. No amplification of unspecific products was observed.

IMMUNOCYTOCHEMISTRY AND ELISA

Alginate beads were fixed using 4% paraformaldehyde, washed with PBS and embedded in OCT compound (Sakura; Zoeterwoude, Netherlands). Sections (7 μm) were cut at −22°C on a Jung Frigocut 2800E cryostat and mounted on Superfrost plus glass microscope slides (Erie Scientific Company; Portsmouth, Netherlands). Sections were dried for 1 h and stored at −20°C until further use. Each step for the immunolabelling was performed at room temperature. After thawing, non-specific staining was blocked by incubation of the sections with 1% bovine serum albumin (Sigma; St. Louis, USA) for 30 min. Sections were incubated for 4 h with a monoclonal antibody against collagen type II (clone II-II6B3, Developmental Studies Hybridoma Bank, University of Iowa). Sections were then incubated for 2 h with a Cy3-coupled goat anti-mouse secondary antibody (Dianova; Hamburg, Germany). Extensive washing with PBS was performed between each step. All sections were counterstained with DAPI and mounted with mowiol. Negative controls were obtained by omitting incubation with the primary antibody.

The expression of type II collagen was also investigated by ELISA using the native type II collagen detection kit (Chondrex Inc., Redmond, USA). Briefly, collagen was digested by 10 mg/ml pepsin dissolved in 0.05 M acetic acid overnight at 4°C with gentle mixing. Cells were then incubated at 35°C for 30 min with 1 mg/ml pancreatic elastase after that samples were centrifuged and supernatants were diluted with the standard dilution buffer provided by the kit. The ELISA plate was coated with collagen type II antibody as described by the supplier, 100 μl/well of sample were added and incubated for 2 h at room temperature. After washing samples were incubated with streptavidin peroxidase-labeled detection reagent (dilution 1:200) for 1 h at room temperature. Finally, after rinsing plates, color reaction followed though H_2O_2 incubation for 30 min at room temperature, reaction was stopped with 2.5 N sulfuric acid and the OD was read at 490 nm in an ELISA reader (Bio-Tek, KC[4] PowerWave$_x$, BioRad, Munich, Germany).

EXPRESSION AND PURIFICATION OF RECOMBINANT HUMAN BMP-2

Recombinant human BMP-2 was expressed in *Escherichia coli* based on a maltose-binding protein (MBP)-fusion expression vector. The malE fusion vector was constructed from pMAL-c2X (New England Biolabs, Beverly, MA) by removing the factor Xa site with an *Aval*–*Bam*HI restriction digest and replacing them with an annealed pair of synthetic oligonucleotides (5′-GTC CCT CGG GCA TCA CCA TCA CCA TCA TTC TGG TCT GGT ACC ACG CGG ATC CGC GC-3′, 5′-GCG CGG ATC CGC GTG GTA CCA GAC CAG AAT GAT GGT GAT GGT GAT GCC CGA GGG AC-3′) which were digested with the same restriction enzymes and encode for a 6×His tag and a thrombin-binding site (LVPRGS). A gene encoding for the mature human BMP-2 was synthesized as *Bam*HI–*Hin*dIII fragment (GENEART, Regensburg, Germany) and cloned into the *Bam*HI–*Hin*dIII digested vector. IPTG induction of transformed *E. coli* cells which were cultivated in a glucose mineral salt medium results in cytoplasmic overexpression of soluble MBP-fusion. Monomeric MBP–BMP-2 fusion protein was isolated after cell disruption by amylose affinity chromatography and r-BMP-2 was purified to homogeneity after *in vitro* dimerization of the BMP-2 still fused to MBP and protease cleavage of the fusion as described earlier [13].

The mature r-BMP-2 homodimer, generated by the proteolytic removal of the MBP domains, migrates as an approximately 26 kDa protein under non-reducing conditions

and as a 13 kDa protein under reducing conditions in SDS-PAGE and has a purity $>95\%$. NH_2-terminal amino acid sequence analysis of the purified BMP-2 reveals the expected single main sequence corresponding to GSQAKHKQRK.

Endotoxin contamination was <0.02 ng/mg of rh-BMP-2 as determined by the chromogenic kinetic method of the limulus amoebocyte lysate (LAL) assay (Kinetic-QCL-kit, BioWhittaker). Biological activity of the final product was measured by its ability to induce alkaline phosphatase expression in mouse C2C12 promyoblast cells [14]. The ED_{50} for this effect was found in the range of 20–40 nM and is in good agreement with data from literature[15].

STATISTICAL ANALYSIS

All data reported represent the median value $\pm$ range (min and max values) of individual analyses of eleven separate donors. A student's *t* test was performed to compare monolayer vs alginate cultures and to assess the effect of application of r-BMP-2 to monolayer and alginate cultures.

Results

GENE EXPRESSION DIFFERENCES IN CHONDROCYTES FROM DISTINCT DONORS

In the present study we used chondrocytes from 11 individuals. All cartilage biopsies were harvested from low-weight bearing and macroscopically healthy areas of the femoral condyle. The culture protocol was identical for all experiments. Nevertheless, the expression levels of mRNA of several differentiation markers including type II collagen, aggrecan, BMP-2 or IL-1β differed considerably between the individual donors (see Table I) and seem not be related either to gender or age.

EFFECT OF THE CULTURE SYSTEM ON THE CHONDROGENIC PHENOTYPE

Freshly isolated human chondrocytes were first expanded in monolayer for 7–10 days to reach confluence. One aliquot of the cells was used to investigate the transcript levels of these primary culture cells (P0). Remaining cells were split and further cultured in monolayer or embedded in alginate beads (see Fig. 1 for cell culture scheme): one aliquot was seeded in cell culture flasks to further expand the cells for 2 weeks in a first subculture (P1-2D). The third aliquot of cells was seeded in alginate beads and cultured for 2 weeks (P1-3D). Then cells were harvested and gene expression patterns were investigated by quantitative RT-PCR (Fig. 2). The mRNA levels of alginate cultures are reported relative to that of monolayers.

At P1 the expression of type I collagen significantly decreased in chondrocytes cultured in alginate (P1-3D) compared to cells in monolayer (P1-2D, $P<0.001$). Transcripts encoding type II collagen increased in 10 of 11 donors (mean increase: 115-fold; max: 643-fold; min: 1.6-fold) when cells were cultured in alginate. However, in cells from one donor the type II collagen level was not affected by the culture system. The tendency of chondrocytes to produce more type II collagen in 3D cultures compared to monolayer cultures was also confirmed by immunocytochemistry using an antibody specific for collagen type II (Fig. 3). The type II collagen staining is stronger in cells embedded in alginate when compared to cells grown as monolayer [Fig. 3(A–C)] and an almost 10-fold increase in type II collagen synthesis was recorded by ELISA in samples of 3D cultures in comparison to monolayer cultures [$n=4$, 9.45-fold $\pm$ 6.8, $P<0.043$; Fig. 3(D)]. Interestingly, in all donors the transcript levels for BMP-2, a chondrogenic marker, were higher in alginate cultures compared to monolayers. In contrast, the expression of BMP-4 (11/11, $P<0.001$) and IL-18 (10/11, $P<0.05$) were significantly lower in 3D cultures. The culture system had no effect on the expression of aggrecan, IL-1β or IL-10 (Fig. 2).

In a second experiment cells were embedded in alginate after a first subculture on monolayer. Transcript levels obtained for P2 alginate were expressed relative to that obtained for P2 monolayer (Fig. 4). Again, the expression of type I collagen was reduced in all alginate cultures in comparison to P2 monolayer cultures ($P<0.01$) and the transcripts for type II collagen were up-regulated in all donors. The mean increase was of 1000-fold (max: 3120-fold; min: 5-fold). Considerable differences in the magnitude of type II collagen mRNA increases were observed between distinct donors. In two cases we cultured the cells in alginate until P3, to investigate if the expression of type II collagen was further up-regulated in 3D cultures (Fig. 5). The increased amplitude seemed to depend on the original expression level in the primary culture: at P3-3D the

Table I

mRNA expression levels relative to the housekeeping gene GAPDH for chondrocytes from 11 donors in primary culture. Considerable differences in the gene expression are present between the five donors, but they seemed not to be related neither to gender, nor to age

	Gender	Age	mRNA expression levels in primary cultures				
			Collagen type II	Collagen type I	Aggrecan	BMP-2	IL-1β
Donor 1	M	19	1 498.4	96 539.7	17 834.6	38.4	7.9
Donor 2	M	27	15.3	78 175.2	21 806.6	621.7	1.4
Donor 3	M	30	189.4	165 097.1	14 123.6	22.9	0.3
Donor 4	F	20	131.8	139 610.0	13 294.5	278.8	4.6
Donor 5	F	23	5 822.5	413 536.9	32 592.3	233.3	0.1
Donor 6	F	31	512.7	172 027.0	20 792.1	2 599.0	0.1
Donor 7	M	38	1 042.0	137 944.0	12 672.0	1 555.0	0.1
Donor 8	F	71	1 294.0	161 481.0	17 232.0	2 269.0	0.1
Donor 9	M	32	217.9	179 076.0	29 317.3	14.6	2.1
Donor 10	M	77	37 297.1	56 978.7	73 141.0	24 462.9	1.1
Donor 11	M	70	15 774.5	96 153.9	40 693.0	971.9	1.3

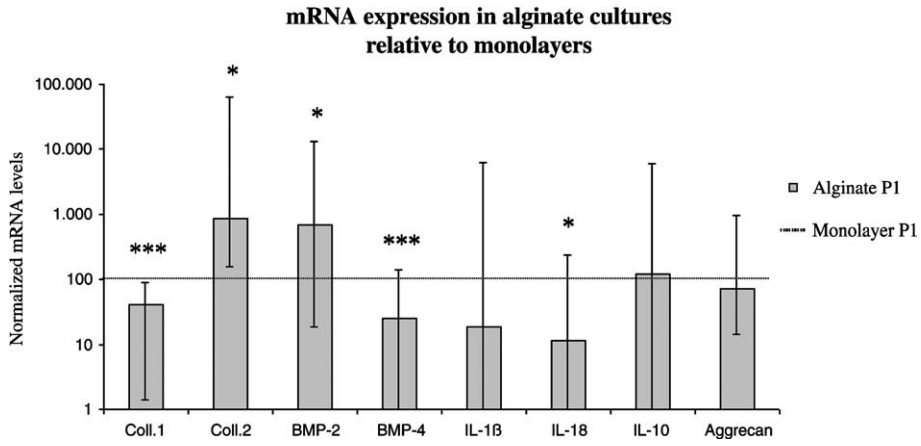

Fig. 2. Effect of culture conditions on matrix protein and cytokine gene expression in first subcultures of human articular chondrocytes. Chondrocytes from each donor were cultured individually in monolayer and in alginate beads for 2 weeks. The transcript levels in alginate cultures are expressed relative to that of monolayers. The mRNA levels for all genes were normalized with the amount of the housekeeping gene GAPDH. Values indicate the median result of five experiments; deviations are represented by the range between the minimal and the maximal values observed. Statistical significance is given as follows:***$P<0.001$; *$P<0.05$.

cells re-expressed the same amount of collagen type II as at P0.

The influence of culture system at P2 on the other transcripts was similar to that observed at P1. BMP-2 was mRNA up-regulated in all cases and transcripts encoding BMP-4 and IL-18 were significantly down-regulated in alginate cultures ($P<0.001$ and $P<0.05$, respectively). The expression of IL-18 is high in primary cultures, decreased during different passages in monolayer and remained low in alginate. Similar to what was seen at P1, IL-1β and aggrecan were not influenced by the culture system.

EFFECT OF RH-BMP-2 ON THE CHONDROGENIC PHENOTYPE IN DIFFERENT CULTURE SYSTEMS

Next, chondrocytes were expanded in 2D and 3D cultures in absence or presence of 100 ng/ml hr-BMP-2 on monolayers [Fig. 6(A)] and on alginate cultures [Fig. 6(B)]. Incubation of chondrocytes in 2D culture in presence hr-BMP-2 did not change the transcript patterns sizably for most genes when compared to untreated controls [Fig. 6(A)]. In 8/11 samples application of hr-BMP-2 induced a slight increase of the type II collagen transcript amounts (mean: 11.3-fold; max: 76.7-fold; min: 1.3-fold, $P<0.04$).

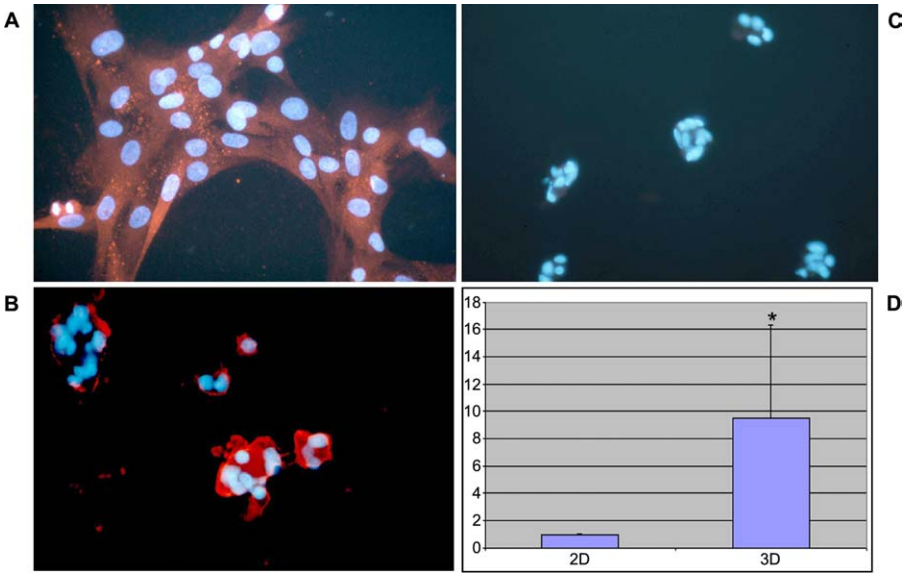

Fig. 3. Immunocytochemical localization of collagen type II in primary cultures. (A) Collagen type II staining in monolayers. (B) Collagen type II staining in cells embedded in alginate beads. (C) Negative control of alginate culture obtained by omitting the primary antibody. All slides were counterstained with DAPI. Original magnification: ×40. (D) Detection of type II collagen expression by ELISA in chondrocytes embedded in alginate beads compared to cells grown in monolayer cultures ($n=4$; *$P<0,05$).

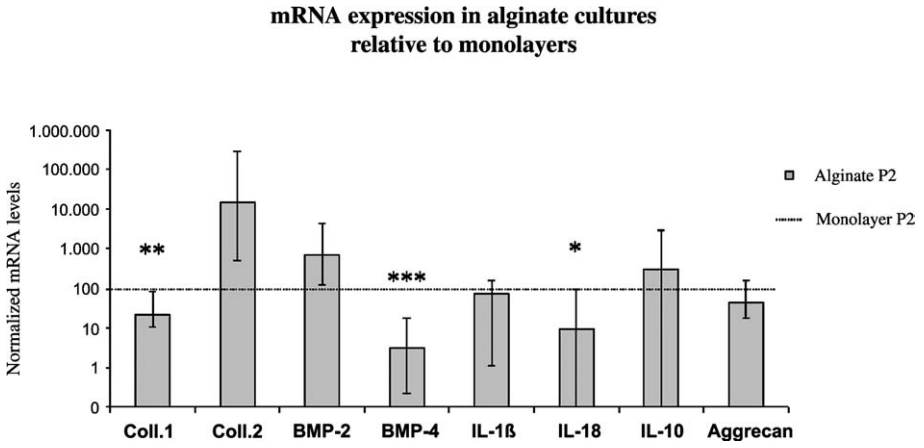

Fig. 4. Effect of culture conditions on matrix protein and cytokine gene expression in second subcultures of human articular chondrocytes. The transcript levels in alginate P2 cultures are expressed relative to that of P2 monolayers. The mRNA levels for all genes were normalized with the amount of the housekeeping gene GAPDH. Values indicate the median result of five experiments; deviations are represented by the range between the minimal and the maximal values observed. Statistical significance is given as follows: $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$.

A more prominent effect was seen when cells were encapsulated in alginate beads in presence of 100 ng/ml hr-BMP-2 [Fig. 6(B)]. The hr-BMP-2 induced a significant increase in type II collagen encoding mRNA in [8/10 samples, mean: 256-fold; max: 1350-fold; min: 1.2-fold, $P<0.05$; Fig. 6(B)]. An enhanced type II collagen expression was also noted by ELISA [Fig. 6(C)]. Addition of BMP-2 to chondrocytes in alginate beads almost doubled in 3/4 samples the type II collagen expression (11.94-fold $\pm$ 8.57) when compared to the respective 3D controls [5.27-fold $\pm$ 5.7; Fig. 6(C)]. In one sample, addition of BMP-2 did not enhance type II collagen expression (data not shown). Human r-BMP-2 had no effect on the collagen type II protein level in monolayer.

In contrast to 2D cultures, in 3D cultures hr-BMP-2 significantly reduced IL-1β encoding mRNA amounts [10/10, mean: 0.63-fold; min: 0.06; max: 1, $P<0.003$, Fig. 6(B)]. Moreover, aggrecan mRNA was significantly up-regulated through the application of hr-BMP-2 in alginate cultures (9/10 samples; mean: 3.2-fold; max: 9-fold; min: 1-fold). Similar to what was observed in monolayers, r-BMP-2 seemed not to influence the transcript levels encoding type I collagen, BMP-2 and BMP-4, IL-18 and IL-10.

Discussion

Cartilage stabilization and repair constitute a major challenge in joint diseases. Autologous chondrocyte transplantation (ACT) is a promising technique for repair of large cartilage defects in joints. The experience shows, that the quality of the cells implanted into the defect is of central importance for the therapeutic success of ACT[6]. The chondrogenic phenotype must be preserved during the period of *in vitro* cell expansion. Seeding cells in alginate beads supports chondrogenesis and maintains a chondrogenic phenotype of the cells. In beads chondrocytes synthesize cartilage matrix components similar to those present in the native tissue[8,16]. In addition, there is good evidence that alginate possesses the physical characteristics and handling properties required to support cells and serve as a carrier for treatment of full-thickness defects *in vivo*[17,18].

In the present study, two distinct cell culture systems and a morphogen of the TGF-β family, BMP-2, were analysed for their potential to maintain or enhance chondrocyte differentiation and cartilage maturation in an artificial model of human cartilage tissue engineering. In the first part of this study we examined the effect of the cell culture system on

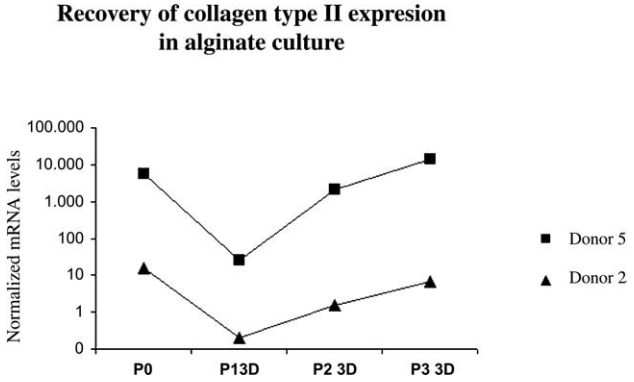

Fig. 5. Recovery of collagen type II expression in alginate culture. Chondrocytes from two distinct human donors were analysed after 7–10 days of expansion in monolayer as primary cultures (P0) and after incubation in alginate beads (3D) for 2 weeks in P1, P2 or P3. A progressive recovery of the type II collagen expression was observed. At P3 the expression levels reached those observed in the primary culture. The mRNA levels for type II collagen were normalized with the amounts of housekeeping gene GAPDH.

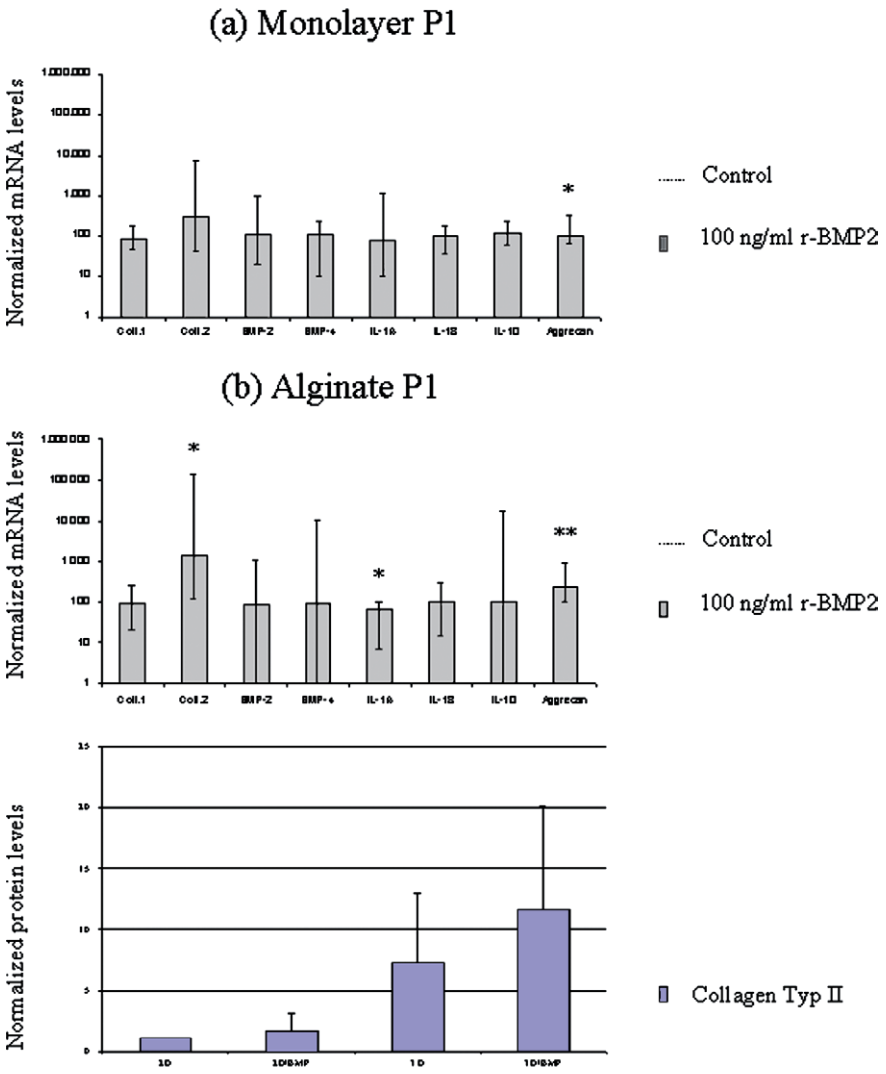

Fig. 6. Effect of hr-BMP-2 on matrix protein and cytokine gene expression in first subcultures of human articular chondrocytes The mRNA levels of cells in presence of 100 ng/ml BMP-2 in monolayer (A) or alginate bead cultures (B) are expressed relative to that of control cells without BMP-2. The mRNA levels for all genes were normalized with the amount of the housekeeping gene GAPDH. Values indicate the median result of four experiments; deviations are represented by the range between the minimal and the maximal values observed. Statistical significance is given as follows: $^{**}P<0.01$; $^{*}P<0.05$. The type II collagen expression at the protein level in 2D vs 3D cultures in absence or presence of 100 ng/ml hr-BMP-2 was investigated by ELISA (C). The data represent the median collagen induction index ($n = 3$ samples) normalized to 2D chondrocytes without hr-BMP-2.

the chondrogenic phenotype. We found that when P1 chondrocytes from healthy human donors were cultured in alginate beads, phenotype markers for cartilage, such as type II collagen and BMP-2, were significantly up-regulated while the expression of type I collagen was significantly down-regulated when compared to monolayer cultures. The maintenance of the chondrogenic phenotype was also preserved in cells which were passaged twice, before being encapsulated in alginate beads. Interestingly, the alginate system significantly decreased the expression of IL-18 in P1 and P2 chondrocytes. This inflammatory cytokine has been shown to have similar but milder effects as IL-1β. *In vitro* IL-18 can reduce the chondrocytes proliferation rate, which would represent a problem when expanding cells for tissue engineering or transplantation. In addition, IL-18 may contribute to the degradation of cartilage extracellular matrix by inducing stromelysin and by stimulating the production of NO-radicals [19,20]. Our results corroborate previous work [7,8,21–23], indicating that alginate is an interesting culture system to maintain chondrocytes in a differentiated stage or to induce a process of re-differentiation. However, growth of cells in alginate alone may not be sufficient for cell expansion for the purpose of tissue engineering, therapeutic cell culture or medical cartilage repair, because of their very low proliferation rate when embedded in alginate beads[24].

Chondrocytes were expanded in monolayer cultures or embedded in alginate in presence or absence of 100 ng/ml hr-BMP-2. The potential clinical uses of proteins from the TGF-β family in treating bone and cartilage defects have stimulated extensive research[25–27]. In addition, BMP-2 has been shown to play a role in the regulation of cartilage-related genes[27,28]. In contrast to previous reports[28], we could not observe a significant improvement of the chondrogenic phenotype through application of 100 ng/ml

hr-BMP-2 on chondrocytes expanded in monolayer culture. One possible explanation could be that human chondrocytes grown under Good Manufacturing Practice (GMP) conditions suitable for ACT in monolayer are not responsive to BMP-2. In contrast, chondrocytes of other species such as the equine chondrocytes used in those studies and expanded under experimental conditions including xenobiotic serum[28] may react differently.

In comparison to the 2D cultures, the situation changed when cells were cultured three-dimensionally in the presence of hr-BMP-2: the quality of the cells was still increased compared to control alginate cultures. Application of hr-BMP-2 on alginate cultures induced a statistically significant increase of the collagen type II expression ($P \leq 0.05$) as well as a significant increase in the aggrecan expression ($P \leq 0.01$). This effect might be explained by ionic interactions between the basic residues in the heparin-binding site of hr-BMP-2 and carboxylate groups of alginic acid which contribute to a restricted diffusion of the BMP[29].

In conclusion, our results indicate that alginate encapsulation is efficient not only in causing the re-expression or maintenance of chondrogenic markers in passaged human chondrocytes and in reducing the expression levels of dedifferentiation markers, but it also represents an interesting cell culture model because of its increased responsiveness of human chondrocytes to growth factor BMP-2. This could be of tremendous use for tissue engineering purposes. In addition, alginate has a major advantage over most other systems in that the cells can be recovered easily in a healthy state by simply dissolving the calcium alginate gel with chelating agents. It is even imaginable to think about implanting alginate constructs directly into a cartilage defect. Such experiments were already performed in mice[17] and in rabbits[18] resulting in a good cartilage formation. However, as our data do not corroborate results obtained with animal cells[18], the use of alginate as scaffold for ACT in humans must be considered with outmost care. Further, it is still unclear, whether alginate-augmented cartilage tissue could induce immune reactions *in vivo* as discussed recently[30].

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