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Molecular analysis of expansion, differentiation, and growth factor treatment of human chondrocytes identifies differentiation markers and growth-related genes☆

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Abstract

This study is intended to optimise expansion and differentiation of cultured human chondrocytes by growth factor application and to identify molecular markers to monitor their differentiation state. We dissected the molecular consequences of matrix release, monolayer, and 3D-alginate culture, growth factor optimised expansion, and re-differentiation protocols by gene expression analysis. Among 19 common cartilage molecules assessed by cDNA array, six proved best to monitor differentiation. Instant down-regulation at release of cells from the matrix was strongest for COL 2A1, fibromodulin, and PRELP while LUM, CHI3L1, and CHI3L2 were expansion-related. Both gene sets reflected the physiologic effects of the most potent growth-inducing (PDGF-BB) and proteoglycan-inducing (BMP-4) factors. Only CRTAC1 expression correlated with 2D/3D switches while the molecular phenotype of native chondrocytes was not restored. The markers and optimised protocols we suggest can help to improve cell therapy of cartilage defects and chondrocyte differentiation from stem cell sources.

Keywords: Cartilage; Proteoglycan; Collagen; Tissue engineering; Bone morphogenic proteins; 3D-culture

Hyaline cartilage is an avascular deformable tissue that covers articulating surfaces and protects them against the damaging effect of decades of repetitive load and friction associated with joint movement. For the investigation of cartilage biology several in vitro culture models have been developed. These include monolayers, three-dimensional culture systems like alginate culture or high density pellet culture, and explant models [1,2]. There has been growing interest in such culture models, since tissue engineering of cartilage and newly developed cell therapeutic approaches like autologous chondrocyte transplantation (ACT) rely on chondrocytes expanded in tissue culture [3,4]. For ACT small cartilage biopsies are taken from less loaded regions of a patient's joint. The embedded chondrocytes are released from the extracellular matrix by enzymatic digestion and expanded in vitro to obtain high cell numbers. Finally these cells are transplanted to a focal cartilage defect in the same joint to enhance the extremely limited regeneration capacity of articular cartilage [5].

The proliferative capacity of chondrocytes is substantially higher under monolayer conditions compared to non-adherent systems. For this reason monolayers are the chosen culture models for expansion of chondrocytes, although alterations in cell morphology and matrix gene expression compared to three-dimensional models and native cartilage have been documented [6–8]. Changes include loss of the rounded cell shape, loss of collagen type II and aggrecan core protein expression, and loss of the capacity to induce stable cartilage implants after intramuscular injection into nude mice [9]. Growth factors are useful to manipulate chondrocytes in culture and may be used to promote growth or improve the maintenance and even re-expression of the chondrocyte phenotype [10]. Most studies addressing the

☆ *Abbreviations*: BMP-2, -4, -6, -7, bone morphogenetic protein 2, 4, 6, 7; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; GDF-5, growth and differentiation factor 5; IGF-I, insulin-like growth factor I; PDGF-BB, platelet derived growth factor BB; TGF-β_1, transforming growth factor β_1; PCR, polymerase chain reaction; RT, reverse transcriptase.

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molecular effects of chondrocyte culture and growth factor treatment, however, compared the effects of only few single growth factors and studied the gene expression of only few genes like collagen I, collagen II, aggrecan, and fibronectin [11–13]. The aim of this study was to optimise culture conditions for expansion and redifferentiation of human chondrocytes and to dissect the molecular consequences of matrix release, which switches between 2D- and 3D-culture and growth factor treatment to identify markers that allow monitoring of expansion and differentiation and thus comparison and optimisation of cell cultures for in vivo applications. Ten distinct growth factors and selected combinations of them were compared by ^{3}H-thymidine labelling of newly synthesised DNA and detection of proteoglycan synthesis by $^{35}SO_4$ incorporation into chondrocytes. Gene expression of 19 common cartilage expressed molecules was quantified with a custom-made cartilage cDNA array.

Materials and methods

Materials. For all experiments human recombinant growth factors were used. GDF-5 was a gift from Biopharm (Heidelberg, Germany). EGF, bFGF, and TGF-β_1 were purchased from Strathmann Biotech (Hamburg, Germany), PDGF-BB, IGF-I, and TGF-β_3 from Sigma–Aldrich (Deisenhofen, Germany). BMP-2, BMP-4, BMP-6, and BMP-7 were supplied by Genetics Institute (Madison, USA).

Human samples. Cartilage samples from peripheral, less loaded regions of knee joints were obtained after informed consent from 30 patients undergoing total joint replacement. The study was approved by the local ethics committee.

Chondrocyte isolation and culture. Cartilage slices outside of regions with macroscopic evident degeneration were removed from the underlying bone, minced with a scalpel, and digested with 1.5 mg/ml of collagenase B (Roche, Mannheim, Germany) and 0.1 mg/ml of hyaluronidase (Serva, Heidelberg, Germany) overnight (18 h) at 37 °C. The released cells were plated in 80 cm^2 cell culture flasks in Dulbeccos modified Eagle's medium (DMEM (Life Technologies, Karlsruhe, Germany)) supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and maintained in a humidified atmosphere at 6% CO_2 and 37 °C. The medium was replaced twice a week. Confluence was usually achieved after 2–3 weeks.

Alternatively, the isolated cells were encapsulated in alginate beads at a density of 1×10^6 cells/ml of gel as described [14]. Briefly, cells were suspended in a sterile 0.15 M NaCl containing low viscosity alginate gel (1.2%) and then slowly pressed through a 22 gauge needle in a dropwise fashion into a 102 mM $CaCl_2$ solution. After instantaneous gelation the beads were allowed to polymerise for 10 min. Washed beads were maintained in a complete culture medium in a humidified atmosphere at 6% CO_2 and 37 °C. The medium was replaced twice a week. For chondrocyte recovery, the washed beads were dissolved in 1 ml of 55 mM sodium citrate and 0.15 M NaCl, pH 6.5. The solubilised alginate was centrifuged for 10 min at 150*g* and cells were used for RNA isolation.

Stimulation with growth factors. Cells from primary monolayer cultures (P_0) were plated at 5.000 cells/well into 96-well plates or at 200.000 cells/well into 6-well plates and cultured for 4–6 days in the presence of various human recombinant growth factors (BMP-2, BMP-4, BMP-6, BMP-7, IGF-I, EGF, TGF-β_1, bFGF, GDF-5, and PDGF-BB) in different concentrations.

Proliferation assay. Cell proliferation was determined by ^{3}H-thymidine incorporation. For the final 18 h of a 4-day incubation period [Methyl-^{3}H]thymidine (0.25 μCi/well) was added before cells were detached by trypsin/EDTA, harvested onto a filter mat, and the incorporated radioactivity (counts per minute, cpm) was determined in a Microbeta-counter (Wallac ADL, Turku, Finland). Each sample was tested in triplicates and the mean of the growth factor-treated samples was referred to the mean of the untreated control cultures.

Proteoglycan synthesis. Quantification of the proteoglycan synthesis was performed using a modified protocol according to Masuda et al. [15]. Proteoglycans were labelled by incubating the cells during the last 18 h of the assay (6 days) in a medium containing 20 μCi/ml [^{35}S]sulphate. After extraction with guanidine/HCl in the presence of protease inhibitors the proteoglycans were complexed to alcian blue and precipitated on filter plates. After extensive washing the incorporated [^{35}S]sulphate (cpm) on the filter plate was quantified in a Microbeta-counter. The mean of triplicates of the growth factor-treated samples was referred to the mean of the untreated control cultures.

RNA isolation. Total RNA was isolated from cultured cells using a standard guanidinium thiocyanate/phenol extraction (peqGOLD RNA Pure; Peqlab, Erlangen, Germany). Polyadenylated mRNA was isolated using oligo(dT) coupled to magnetic beads (Dynabeads; Dynal, Oslo, Norway) according to manufacturer's instruction. Cartilage mRNA was obtained directly from the shock-frozen and homogenised (Dismembrator S, Braun Biotech, Melsungen, Germany) tissue using oligo(dT) coupled to magnetic beads.

cDNA array production. Selected cDNA fragments of 19 human genes predominantly expressed in articular cartilage (Table 1), two housekeeping genes, and as negative controls three genes from *Arabidopsis thaliana* were cloned into pBluescript SK$^+$ vector (Stratagene, Amsterdam, Netherlands). cDNAs were PCR amplified using vector-specific primers and 50 ng of plasmid as template. PCR products were purified (PCR purification kit, Qiagen, Hilden, Germany), concentrated and standardised amounts were arrayed onto positively charged nylon filters (Hybond N$^+$, Amersham Pharmacia Biotech, Freiburg, Germany). All cDNAs were spotted twice on each filter.

cDNA array hybridisation. Human RNA samples were labelled with ^{32}[P]-dATP by gene-specific cDNA synthesis according to manufacturer's protocol (SuperScript II, Life Technologies). The cDNA probes were denatured and hybridised to the membranes overnight at 68 °C in 0.5 M phosphate buffer, pH 7.2/7% SDS. The membranes were washed three times for 30 min in 0.04 M phosphate buffer, pH 7.2/1% SDS at 68 °C and were exposed to a phosphor plate for up to 72 h. Images were captured on a Bio-Imaging Analyser BAS-1800 II using BAS Reader 2.26 beta software (Fuji/Raytest, Straubenhardt, Germany) and analysed using the AIDA software (Fuji/Raytest). Direct comparisons between expression profiles were based on the emission in PLS/mm^2, which corresponds to the optical density (luminance, PLS count) related to the area of emission, measured for each hybridisation dot. The relative level of expression for each gene was calculated as a mean value of the normalised signal intensities (duplicates) and gene intensity values were normalised to the housekeeping gene controls on the same filter (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cytoplasmic beta-actin (ACTB) (cartilage cDNA arrays)).

cDNA synthesis and LightCycler real-time PCR. First-stranded cDNA was generated from mRNA by using reverse transcriptase (SuperScript II, Life Technologies) and oligo(dT)-primers. To quantify the mRNA levels with the LightCycler (Roche Diagnostics, Mannheim, Germany) aliquots of the first-stranded cDNAs were amplified and real-time fluorimetric intensity of SYBR green I was monitored. The $MgCl_2$ and the cycling parameters were optimised according to the LightCycler protocol (LightCycler Operator's Manual, Version 3.5, October 2000, Roche). The following primers were used for amplification: GAPDH-for, 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′; GAPDH-rev, 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′; FMOD-for, 5′-ATG TAC TGT GAC AAT CGC AAC-3′; FMOD-rev

Table 1
Genes included in the cartilage cDNA array

Gene	Approved symbol	GenBank accession no.
Actin, beta	ACTB	X00351
Aggrecan 1	AGC1	M55172
Biglycan	BGN	J04599
Cartilage acidic protein 1 (CEP-68)	CRTAC1	AK001182
Cartilage intermediate layer protein	CILP	AF035408
Cartilage linking protein 1	CRTL1	U43328
Cartilage oligomeric matrix protein	COMP	L32137
Cartilage-derived morphogenetic protein-1	CDMP1	U13660
Chitinase 3-like 1 (cartilage glycoprotein-39; YKL-40)	CHI3L1	M80927
Chitinase 3-like 2 (YKL-39)	CHI3L2	U58514
Chondroitin sulphate proteoglycan 2 (versican)	CSPG2	U16306
Chondromodulin I precursor	CHM-1	AB006000
Collagen, type II, alpha 1	COL 2A1	X16468
Collagen, type IX, alpha 2	COL 9A2	M95610
Collagen, type XI, alpha 1	COL 11A1	J04177
Decorin	DCN	M14219
Fibromodulin	FMOD	U05291
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	X01677
Lumican	LUM	U18728
Melanoma inhibitory activity (CD-RAP)	MIA	X84707
Proline arginine-rich end leucine-rich repeat protein	PRELP	U29089

5′-GAA GTT CAC GAC GTC CAC C-3′, PRELP-for, 5′-CCA ACA AGA CGA CCA AGA CC-3′; PRELP-rev, 5′-CTG GTG AAT GGC GGT GGG-3′; COL 2A1-for, 5′-TGG CCT GAG ACA GCA TGA-3′; COL 2A1-rev, 5′-AGT GTT GGG AGC CAG ATT G-3′; LUM-for, 5′-ACC AGA TTG ACC ATA TTG ATG A-3′; LUM-rev, 5′-GGA CAG ATC CAG CTC AAC C-3′; CHI3L1-for, 5′-CCA ATA TAA GCA ACG ATC ACA T-3′; CHI3L1-rev, 5′-TCC AAC ACC AGT CTC AGA AG-3′; CHI3L2-for, 5′-GGC AGG TGT AGT GGT CTT G-3′; CHI3L2-rev, 5′-GCA TAT TCC ACA TTG TAG TAG G-3′. These primer pairs result in PCR products of 600 bp (GAPDH), 674 bp (PRELP), 373 bp (COL 2A1), 559 bp (LUM), 650 bp (CHI3L1), and 708 bp (CHI3L2). The concentration of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control for input RNA because it is considered a stable housekeeping gene. The GAPDH concentration was determined once for each cDNA sample and used to normalise all other genes tested from the same cDNA sample. The copy ratio of each analysed cDNA was determined as the mean values of three experiments.

Statistics. All samples were determined in triplicates. Median values of treated cell were referred to the value of the untreated controls (stimulation index, SI). Data of n different primary cultures were expressed as means of stimulation indices ± standard deviation and compared by one-sample t test; $p < 0.05$ was considered significant.

Results

Altered gene expression in chondrocytes released from extracellular matrix

A custom-made cartilage cDNA array was developed to allow simultaneous detection and reliable quantification of the gene expression levels of 19 cartilage molecules with special focus on extracellular matrix components (Fig. 1A). Freshly isolated chondrocytes released from the extracellular matrix by collagenase digestion revealed major changes in gene expression compared to native chondrocytes (Fig. 1A). Averaged data of several experiments demonstrated that especially FMOD and PRELP were rapidly down-regulated more than 5-fold (Fig. 1B); expression of collagen II alpha 1 (COL 2A1), BGN, and AGC1 was reduced more than 3-fold. The expression levels of these genes remained low when the cells were cultured in monolayer for one week (Fig. 1B) and persisted or decreased further during long time culture (up to 8 weeks) (data not shown). In contrast, matrix depletion reduced the expression of collagen XI alpha1 (COL 11A1), MIA, CRTAC1, and others by about 2-fold, while RNA levels of LUM, CHI3L1, and CHI3L2 remained unaffected. Expression of the two chitinase 3-like molecules CHI3L1 and CHI3L2 together with LUM increased during in vitro expansion for one week in monolayer culture (Fig. 1B) and raised further during prolonged culture (data not shown). Alteration of gene expression of selected genes was confirmed by quantitative RT-PCR using the LightCycler technology (Fig. 1C). The data demonstrate that reliable quantification of gene expression levels was obtained by cDNA array analysis. In sum, drop of differentiation markers like FMOD, PRELP, and COL 2A1 at release from the cartilage matrix was followed by an up-regulation of the expansion-stimulated genes LUM, CHI3L1, and CHI3L2 during monolayer culture.

Alginate culture cannot restore cartilage-like expression profiles

To compare the molecular consequences of 2D- versus 3D-culture, chondrocytes were seeded either in alginate

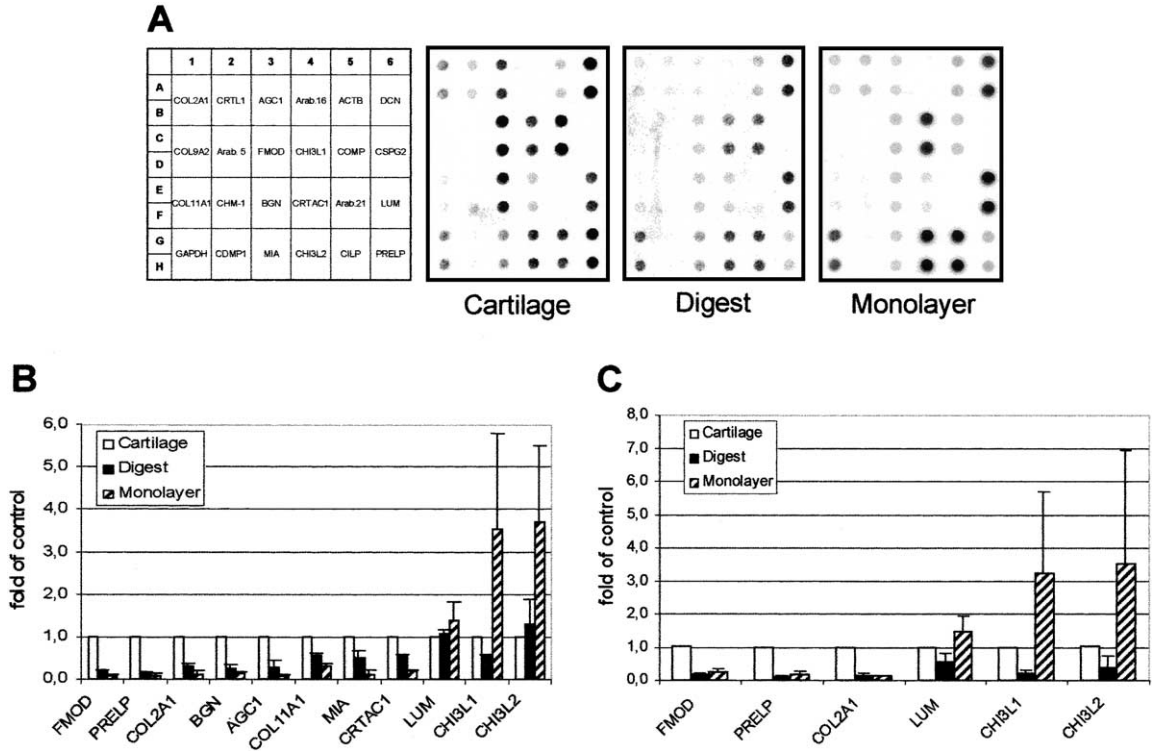

Fig. 1. Gene expression levels of chondrocytes in native articular cartilage, after matrix release, and in monolayer culture: (A) PCR amplified fragments of common cartilage expressed molecules ($n = 19$), housekeeping genes (GAPDH and ACTB), and negative controls (3 Arabidopsis genes) were immobilised on the cartilage cDNA array (see Materials and methods). Cartilage biopsies were minced and used for RNA isolation (Cartilage) or enzymatic digestion overnight, respectively. One half of the released chondrocytes was used for RNA isolation (Digest), the other half was cultured in monolayer for one week before RNA was isolated (Chondrocytes). The different RNA samples were used to generate ^{32}P-labelled first-strand cDNA probes which were hybridised with the cartilage cDNA array (one of the three similar experiments is shown). (B) Quantitative analysis of gene expression levels of selected genes. The signal intensities were normalised according to the housekeeping genes on each filter. Mean values of three independent experiments are shown and expressed as relative values compared to expression in cartilage (assigned a value of 1.0). Bars represent standard deviations. (C) Confirmation of the gene expression levels by real-time PCR. Expression of selected genes was normalised to the corresponding GAPDH expression level in the same sample. Mean values of three independent experiments are shown and expressed as relative values compared to the expression in cartilage (assigned a value of 1.0). Bars represent standard deviations.

beads (1×10^6 cells/ml) or in monolayers (1×10^5 to 1×10^6 cells/ml) immediately after release from the matrix (Fig. 2A). After four weeks in culture the early strong down-regulation of many extracellular matrix molecules, including FMOD, PRELP, and COL 2A1, persisted in 2D- as well as in 3D-culture. In addition, the up-regulation of CHI3L1, CHI3L2, and LUM observed in monolayer chondrocytes was rather stronger when the cells were kept in alginate. This situation did not change when cells initially kept in monolayers were switched to alginate culture for further four weeks. Only the expression of one molecule, the recently described CEP-68 gene (renamed CRTAC1) [20], reversibly correlated with switches from monolayer to alginate culture as confirmed by quantitative RT-PCR (Fig. 2B) (4-fold increase compared to monolayer, 40% of the initial value in cartilage). None of the other analysed genes reversed towards expression levels of native chondrocytes when cells were cultured in alginate beads at similar cell densities as in monolayer. According to quantitative RT-PCR, the complete loss of COL 2A1 expression at four weeks was, however, prevented when the chondrocytes were kept in 3D instead of monolayer culture (Fig. 2B). In sum, alginate culture-induced preservation of a rounded cell morphology and anchorage independent growth did, thus, not correlate with a differentiated chondrocyte phenotype on the molecular level, although complete loss of differentiation markers may be prevented or delayed in this culture form.

Growth stimulation of primary human chondrocytes

To improve in vitro culture of primary human chondrocytes, the effects of 10 human recombinant growth factors were tested in monolayer culture. We analysed BMP-2, -4, -6, -7, IGF-I, EGF, TGF-β_1, bFGF, GDF-5, and PDGF-BB on chondrocytes derived from 12 different donors (mean age 59 years, range 16–79 years) at concentrations of 1, 10, and 100 ng/ml. None of the factors stimulated growth at 1 ng/ml. A

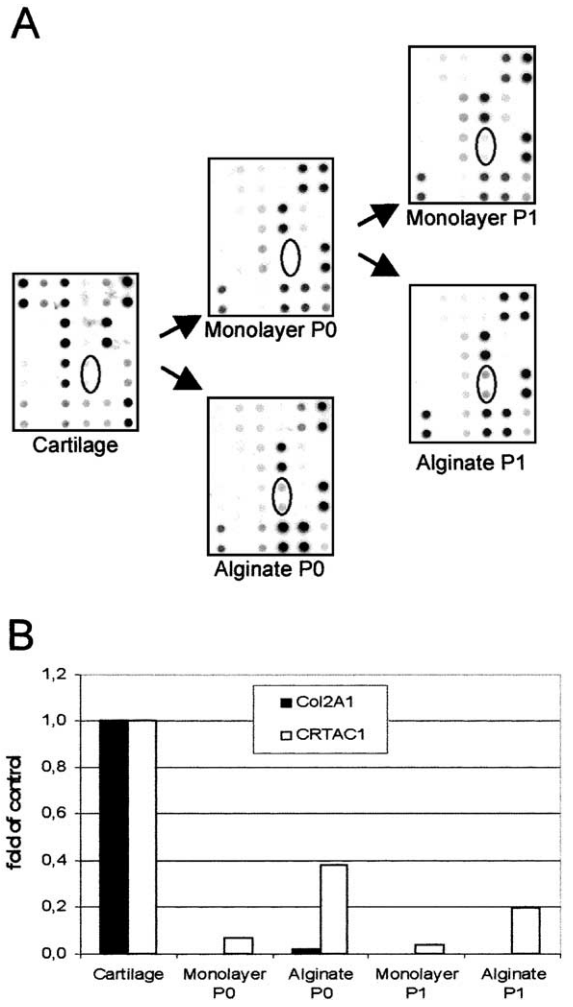

Fig. 2. Comparison of gene expression in articular cartilage and chondrocytes cultured in monolayer or in alginate beads: (A) One part of a cartilage biopsy was used for direct RNA isolation (Cartilage) and the other part was digested overnight with collagenase and hyaluronidase. The released cells were cultured in monolayer (Monolayer) or in 3D-alginate-culture (Alginate). After four weeks (P_0) cells were detached from the plastic or released from the alginate matrix and RNA was isolated immediately. Part of the cells from monolayer cultures were kept for additional four weeks (P_1) in monolayer or alginate beads, respectively. Isolated RNA samples were used to generate ^{32}P-labelled first-strand cDNA probes which were hybridised on the array to quantify gene expression levels. One of the three similar experiments is shown. () indicates location of CRTAC1 on the cDNA array. (B) Quantification of the gene expression levels by real-time PCR. Expression levels of COL 2A1 and CRTAC1 obtained by LightCylcer analysis were normalised according to the corresponding GAPDH expression in the relevant sample. Mean values of three independent experiments are shown and expressed as relative values compared to the expression in cartilage (assigned a value of 1.0).

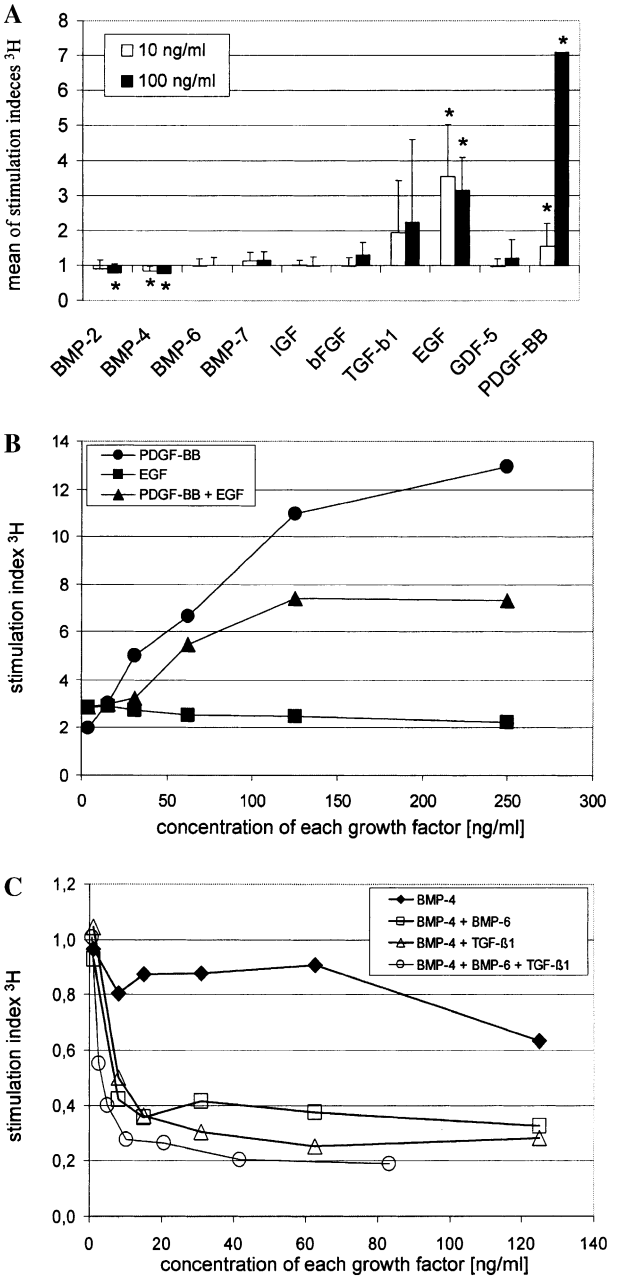

Fig. 3. Effects of growth factors on the proliferation of primary human chondrocytes: (A) 5.000 cells/well were seeded in 96-well plates and cultured for four days in the presence of different growth factors. De novo DNA synthesis was determined by addition of [Methyl-^{3}H]thymidine for the final 18 h. The incorporated radioactivity of triplicate samples was determined. Averaged data represent thymidine incorporation of growth factor-treated cultures referred to growth factor-free controls from $n = 12$ primary cultures. Bars represent standard deviations, * significantly different from corresponding control: $p < 0.05$. (B,C) Dose response curves of selected growth factors and factor combinations. DNA synthesis of growth factor-treated cells was determined as described in (A) and referred to the DNA synthesis of untreated controls (one of the three similar experiments is shown).

significant stimulation of cell expansion was evident for PDGF-BB (7-fold, $p = 0.031$), EGF (3.2-fold, $p = 0.007$) at 100 ng/ml (Fig. 3A), and EGF at a concentration of 10 ng/ml (3.5-fold, $p = 0.044$). In contrast, BMP-4 displayed a significant inhibition of cell proliferation at concentrations of 10 ng/ml (0.85-fold, $p = 0,035$) and 100 ng/ml (0.76-fold, $p = 0,020$), as BMP-2 at 100 ng/ml (0.79-fold, $p = 0,041$) (Fig. 3A). Similar results were obtained when the cells were

cultured under serum-free conditions (data not shown).

PDGF-BB and EGF were combined to further improve cell proliferation (Fig. 3B). At all concentrations tested, addition of EGF reduced the growth stimulatory activity of PDGF-BB and delayed the expansion of the chondrocytes. In contrast, the inhibitory effect of BMP-4 on cell proliferation was, however, potentiated in combinations with BMP-6 and/or TGF-β_1 (Fig. 3C). In conclusion, maximal cell expansion was achieved with PDGF-BB at concentrations $\geqslant 100$ ng/ml while combinations of BMP-4 with BMP 6 and/or TGF-β_1 induced almost a stop of DNA synthesis in the cells.

Stimulation of proteoglycan synthesis by BMP-4

The effect of recombinant growth factors on proteoglycan synthesis was compared at concentrations of 1, 10, and 100 ng/ml by $^{35}SO_4$ incorporation into primary human chondrocytes from six different donors (mean age 67 years, range 55–79 years). BMP-4 showed a significant stimulation of the proteoglycan synthesis at 100 ng/ml (2.1-fold; $p = 0.020$) (Fig. 4A). EGF and PDGF-BB enhanced proteoglycan synthesis parallel to their growth promoting activity (EGF, 1.6-fold, $p = 0.007$; PDGF-BB, 2.5-fold, $p = 0.031$) (Fig. 3A). BMP-2, BMP-6, and TGF-β_1 showed a moderate stimulation of cells from some donors but not from others. In combinations of BMP-4 with TGF-β_1 (Fig. 4B), the stimulatory effect of BMP-4 was completely neutralised by TGF-β_1, resulting in a baseline activity. In contrast, BMP-6 was without any effect on proteoglycan synthesis in combinations with BMP-4 and/or TGF-β_1 suggesting that BMP-4 is the optimal stimulator for induction of proteoglycan synthesis in cultured chondrocytes.

Molecular effects of growth factor treatment

Molecular effects of growth factors on the gene expression levels of common cartilage molecules were analysed by cDNA array analysis (Fig. 5A) and the most potent factors described above were depicted. Addition of PDGF-BB (100 ng/ml) raised the expression levels of LUM (4-fold), CHI3L1 (9-fold), and CHI3L2 (11-fold) above those of normal medium (Fig. 5B) and, thus, stimulated the expansion-related genes even further (Figs. 1B and C). Besides, the expression levels of FMOD and PRELP were slightly changed by PDGF-BB. In contrast, addition of BMP-4 (100 ng/ml) stimulated proteoglycan synthesis, inhibited cell growth, and raised the expression of FMOD (8-fold), PRELP (18-fold), and COL 2A1 (3.5-fold) (Fig. 5B) while LUM, CH13L1, and CH13L2 remained unaffected. In conclusion, up-regulation of differentiation markers indicates

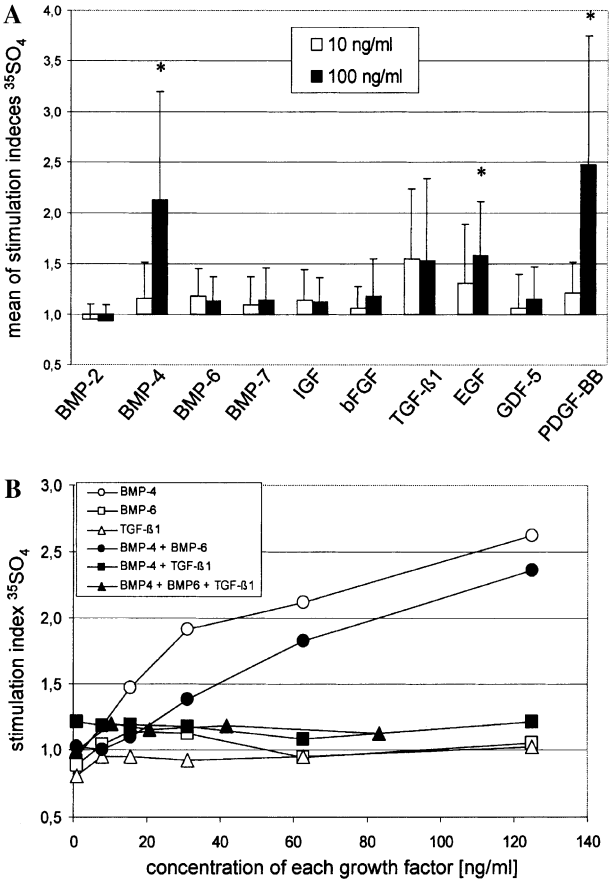

Fig. 4. Effects of growth factors on the proteoglycan synthesis of primary human chondrocytes: (A) 5.000 cells/well were plated in 96-well plates and cultured for six days in the presence of different growth factors. Proteoglycan synthesis was determined by addition of [^{35}S]sulphate for the final 18 h. The incorporated radioactivity of triplicate samples was separated from free label by alcian blue precipitation of proteoglycans. Averaged data represent sulphate incorporation of growth factor-treated cultures referred to growth factor-free controls from $n = 6$ primary cultures. Bars represent standard deviations, * significantly different from corresponding control: $p < 0.05$. (B) Dose response curves of selected growth factors and factor combinations. Proteoglycan synthesis was determined as described in (A) and referred to the proteoglycan synthesis of the untreated controls (one of the three similar experiments is shown).

partial re-differentiation of chondrocytes by a short-time culture in BMP-4. Expression levels characteristic for native cartilage were, however, not achieved.

Discussion

Phenotypic instability of chondrocytes has been consistently observed in monolayer culture. Cells assume a fibroblast-like morphology and cease to synthesise cartilage-specific molecules like collagen 2 and aggrecan in parallel to cell growth [16,17]. To our knowledge this is the first report demonstrating that the release of chondrocytes from cartilage matrix strongly

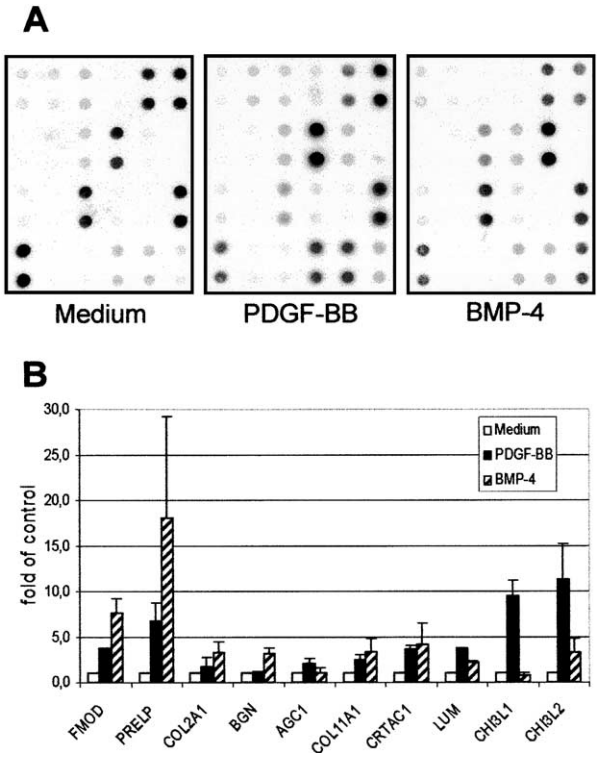

Fig. 5. Gene expression levels after growth factor stimulation: (A) Cartilage biopsies were minced and digested overnight. Cells were plated in culture flasks and grown to confluence before they were seeded at 200.000 cells/well into 6-well plates. After four days in standard culture medium (Medium) or in medium containing either 100 ng/ml PDGF-BB (PDGF-BB) or 100 ng/ml BMP-4 (BMP-4) RNA was isolated and used to generate ^{32}P-labelled first-strand cDNA probes. Gene expression levels were determined using the cartilage cDNA array (one of the three similar experiments is shown). (B) Quantitative analysis of gene expression levels of selected genes. The signal intensities were normalised according to the housekeeping genes on each filter. Mean values of three independent experiments are shown and expressed as relative values compared to the expression in growth factor-free controls (assigned a value of 1.0). Bars represent standard deviations.

affects expression levels of many chondrocyte expressed genes. COL 2A1, FMOD, and PRELP expression decreased most strongly, but also AGC1, COL 11A1, BGN, MIA, and CRTAC1 expression dropped instantly [2] at release from the matrix. Changes aggravated during culture independent of whether the cells were kept in monolayers or 3D-culture. In contrast, the two chitinase 3-like molecules CHI3L1 and CHI3L2 together with LUM remained unaffected by matrix depletion but the expansion phase was characterised by their up-regulation. Thus, the repeatedly reported graduate de-differentiation of chondrocytes to fibroblast-like cells, as judged according to cell phenotype in culture [2,7,8,16,17], started with a strong instant drop of expression levels of many abundant cartilage matrix molecules at release of the cells. This effect may be either due to the loss of the 3D matrix scaffold, the loss of stimulating factors like morphogens embedded in the matrix, the absence of mechanical stimuli, of synovial fluid components or a combination of all. In addition, pharmacological reactions to newly generated matrix degradation products must be kept in mind.

Among 19 common cartilage molecules tested six proved best to monitor the differentiation state of chondrocytes. Since the differentiated phenotype of cells, like that in intact cartilage or under proteoglycan synthesis stimulation correlated with high or up-regulated COL 2A1, FMOD, and PRELP expression levels, we suggest these molecules to monitor differentiation. The expansion state of cells and even more the stimulation of cell growth was characterised by up-regulated CHI3L1, CHI3L2, and LUM levels suggesting them as expansion-related markers. Most convincingly, expression levels of both gene sets reflected the physiologic effects of active growth factors like PDGF-BB and BMP-4. They may, therefore, also be considered response genes for these factors in approaches, where biologic effects need to be monitored, just like COMP expression monitors activity of TGF-β_1 in human synovial cells and articular chondrocytes [18].

Three-dimensional culture systems were reported to support maintenance of a rounded cell shape, allow long-term establishment of a pericellular matrix, and support expression of a differentiated chondrocyte phenotype as judged by COL 2A1 expression [14,19] According to our study, however, chondrocytes in alginate beads did not revert to the molecular phenotype of native chondrocytes in spite of the rounded morphology of the cells and gene expression differences to monolayers were sparse when judged by cDNA array analysis. Evident changes compared to monolayer culture were confined to the CRTAC1 gene [20], whose expression was reversibly down- and up-regulated at switches from 2D- to 3D-culture of the cells. It will, therefore, be interesting to learn more about the possible function of this new potential matrix molecule. In line with our data is a previous report from Kolettas et al. [1] which detected COL 1A1, COL 2A1, COL 9A1, AGC1, and cartilage linking protein in monolayer as well as in suspension culture on mRNA and protein levels and concluded that cell shape does not correlate with the molecular chondrocyte phenotype. In addition, anchorage independent growth and expression of COL 2A1 in 3D-culture were insufficient to predict cartilage formation of chondrocytes after intramuscular injection in nude mice in vivo [9]. Together these data demonstrate that 3D-culture in alginate, although sufficient to switch back cell morphology, is far from being efficient to restore the molecular phenotype on the level of gene expression.

With the intention to optimise growth in culture, we tested 10 recombinant factors and selected combinations

of them for their growth stimulating activity and found two, EGF and PDGF-BB, with significant growth promoting activity in monolayer. Both factors have been known as potent mitogens for many cell types, including chondrocytes, before [21,22]. A lack of additive effects of both factors on chondrocyte growth has, however, not been reported so far and should be further investigated. Although the mean stimulation index of TGF-β_1 was higher than that for PDGF-BB, our data did not reach significance due to high donor variability. This is in contrast to other studies, which suggested the strongest growth stimulation for TGF-β_1 followed by PDGF-AA [23,24] and reported changing responses at older age of the donor [28] and extended duration of subculture [24]. Obviously, also species-specific effects of growth factors on chondrocytes must be considered when data from other mammals [11,22,25–27] and from humans are compared [10,12,21,28]. The cells we analysed were not derived from healthy human cartilage but rather from less affected areas of osteoarthritic joints from patients undergoing knee surgery or arthroscopy. Our study also intended to improve protocols for in vitro expansion of human chondrocytes for cell therapeutic approaches, which are mostly performed with cells harvested from patients with affected joints. Therefore, we averaged the results of all donors, since it will not be feasible to adapt protocols to individual patients in such a setting. Healthy cartilage samples from children ($n = 2$) suggest that the effects of some growth factors may have been underestimated by this way.

In ACT chondrocytes are obtained from cartilage biopsies at less loaded regions of a patient's joint. This procedure means itself an additional injury to the joint surface which should be kept as limited as possible. Expansion in PDGF-BB will allow us to minimise the size of cartilage biopsies and/or reduce expansion time until re-transplantation due to its mitogenic effect on the cells. As a second effect, however, accelerated de-differentiation may be considered in the presence of PDGF-BB as evident from the up-regulation of the expansion-related genes. Inherent cartilage forming capacity of PDGF-treated cells may, therefore, be reduced and should be judged by intramuscular injection into nude mice [9]. As a possible means to enhance the cartilage forming capacity of cultured chondrocytes we suggest stimulation with BMP-4, the optimal proteoglycan-inducing condition in our study. BMP-4 partially reversed the drop in expression of differentiation markers FMOD, PRELP, and COL 2A1, while 3D-culture in standard medium had little effect. This suggests that growth factors have a higher impact on re-differentiation than a switch from 2D- to 3D-culture. So far, the influence of cell expansion and differentiation on the clinical outcome of cartilage repair by ACT [4,29] is unknown and growth factors have not been used to optimise manipulation of chondrocytes dedicated for ACT. Given that in vivo regeneration of cartilage is enhanced by the use of re-differentiated rather than de-differentiated cells, accelerated expansion in PDGF-BB may be followed by induction of re-differentiation by BMP-4 to improve the clinical outcome of cartilage regeneration for the patient. Assessment of the molecular phenotype by cartilage cDNA array or quantitative RT-PCR of the selected marker genes will be powerful tools for quality control of chondrocytes dedicated for transplantation as well as for evaluation of repair tissues which may be accessible at second look biopsies.

At times when old concepts of linear and irreversible cell differentiation are revisited for stem cells [30], the achievement and molecular definition of extreme conditions like maximal expansion and de-differentiation as opposed to improved matrix synthesis, growth arrest, and differentiation may be valuable not only to improve protocols for cell therapy of cartilage or chondrocyte-based tissue engineering. The study we presented defined conditions, identified marker genes, and described a new analytic device which may not only help to improve ACT, but can also aid in advancing chondrocyte differentiation from multipotential cell sources like mesenchymal stem cells. This could promise independence of cartilage biopsies, phenotypic instability of chondrocytes in culture, and the need for two interventions at the same joint.

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