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Preservation of the Chondrocyte's Pericellular Matrix Improves Cell-Induced Cartilage Formation

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ABSTRACT

The extracellular matrix surrounding chondrocytes within a chondron is likely to affect the metabolic activity of these cells. In this study we investigated this by analyzing protein synthesis by intact chondrons obtained from different types of cartilage and compared this with chondrocytes. Chondrons and chondrocytes from goats from different cartilage sources (articular cartilage, nucleus pulposus, and annulus fibrosus) were cultured for 0, 7, 18, and 25 days in alginate beads. Real-time polymerase chain reaction analyses indicated that the gene expression of *Col2a1* was consistently higher by the chondrons compared with the chondrocytes and the *Col1a1* gene expression was consistently lower. Western blotting revealed that Type II collagen extracted from the chondrons was cross-linked. No Type I collagen could be extracted. The amount of proteoglycans was higher for the chondrons from articular cartilage and nucleus pulposus compared with the chondrocytes, but no differences were found between chondrons and chondrocytes from annulus fibrosus. The expression of both *Mmp2* and *Mmp9* was higher by the chondrocytes from articular cartilage and nucleus pulposus compared with the chondrons, whereas no differences were found with the annulus fibrosus cells. Gene expression of *Mmp13* increased strongly by the chondrocytes (>50-fold), but not by the chondrons. Taken together, our data suggest that preserving the pericellular matrix has a positive effect on cell-induced cartilage production. J. Cell. Biochem. 110: 260–271, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: CARTILAGE TISSUE ENGINEERING; CHONDROCYTE; CHONDRON; ALGINATE BEADS

The chondrocyte is the singular cell type in cartilage and is responsible for the maintenance of the extracellular matrix (ECM). The functioning of the cartilage depends entirely on its ECM which is composed mainly of collagens and proteoglycans. As cartilage has a poor ability to repair itself, strategies to regenerate or to engineer this tissue are necessary [Temenoff and Mikos, 2000; Wakitani et al., 2008].

Isolated chondrocytes are often used for cartilage tissue engineering. However, this does not mimic the in vivo situation as in native cartilage the chondrocytes are surrounded by a pericellular matrix, together forming the chondron [Smirzai, 1974]. It has been reported that retention of the native pericellular matrix alters the metabolic activity of the chondrocytes [Larson et al., 2002; Graff et al., 2003]. The pericellular matrix of a chondron is defined primarily by the presence of relatively high levels of Type VI collagen as compared with the interchondron ECM, But it also contains Types II, III, IX, and XI collagen, hyaluronan, proteoglycans such as aggrecan, biglycan, and decorin, and glycoproteins such as fibronectin, link protein, and laminin [Poole et al., 1988, 1990, 1992; Hagg et al., 1998; Keene et al., 1998; Wotton and Duance, 1994].

Essential for cartilage tissue engineering is the maintenance of the differentiated chondrocyte phenotype. Chondrocytes that are grown in monolayer loose their round morphology as they undergo phenotypic changes; they start producing Type I instead of Type II collagen and they have a decreased proteoglycan synthesis [Bonaventure et al., 1994; Stewart et al., 2000]. This protein profile is a characteristic of soft connective tissue rather than hyaline cartilage. Chondrons also dedifferentiate when cultured in monolayer,

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the pericellular matrix is lost, and the chondrocytes spread [Lee and Loeser, 1998]. Maintaining the chondrocytic phenotype can be accomplished by culturing the chondrocytes or chondrons in alginate beads [Häuselmann et al., 1994; Mok et al., 1994; Petit et al., 1996; Lee and Loeser, 1998].

Another important factor with respect to cartilage tissue engineering is matrix integrity. An imbalance between anabolic and catabolic activities can lead to a degenerative process. The pericellular matrix likely plays a role in this, as changes in the pericellular matrix represent one of the earliest identifiable matrix changes associated with cartilage catabolism [Poole et al., 1991; Poole 1997; Lee et al., 2000]. Cell surface receptors can respond to components of the ECM by the production of proteolytic enzymes such as matrix metalloproteinases (MMP).

The aim of this study was to investigate whether preservation of the chondrocyte's native pericellular matrix has a positive effect on cartilage formation. Chondrons and chondrocytes isolated from the chondrons were cultured in alginate beads. The gene and protein expression of Types I and II collagens were monitored and the amount of proteoglycans in the alginate beads was measured. Furthermore, we have examined catabolic factors, such as the gene expression of matrix metalloproteinase 13 (*Mmp13*) and protein expression of Mmp2 and Mmp9. Chondrocytes and chondrons were enzymatically isolated from articular cartilage, nucleus pulposus, and annulus fibrosus from goats. The different types of cartilage were analyzed to investigate whether the effects of retaining the pericellular matrix differ among different cartilaginous tissues.

MATERIALS AND METHODS

HARVESTING OF TISSUES

The cartilaginous tissues were obtained from skeletally mature female Dutch milk goats ($n = 5$) that were used in other studies (the Animal care and use Committee of the VU University Amsterdam approved the use of goats in these studies). After sacrifice, the following cartilaginous tissues were collected: intervertebral disc (from the thoracic part of the spine), and articular cartilage from the knee. The intervertebral discs were divided into the nucleus pulposus and annulus fibrosus.

ISOLATION OF CELLS: CELL CULTURE IN ALGINATE

The tissues were dissected and minced. One half (randomly taken) was used for chondrocyte isolation and the other for chondron isolation.

For chondrocyte isolation, the tissue fragments were subjected to sequential treatments first with Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Paisley, UK) supplemented with 1% foetal bovine serum (FBS; HyClone, Logan, UT), 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2.5 μg/ml of amphotericin B (all from Gibco), and 2.5% (w/v) of pronase E (Sigma, St. Louis, MO) for 1 h, then with DMEM supplemented with 25% FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2.5 μg/ml of amphotericin B, and 0.125% (w/v) of collagenase (CLS-2; Worthington, Lakewood, NJ) for 16 h at 37°C.

For chondron isolation, minced cartilage was digested with 0.3% (w/v) dispase (Gibco) plus 0.2% (w/v) collagenase in phosphate-buffered saline (PBS; Gibco) for 5 h as previously described [Lee et al., 1997].

The cells were filtered through a 70-μm cell strainer (BD Biosciences, San Diego, CA) and washed. The cells that were not directly processed for analysis were resuspended in an alginate solution [1.2% (w/v) Keltone LVCR sodium alginate; Monsanto, San Diego, CA] in physiological salt (0.9% NaCl, 0.2-μm sterile-filtered), creating a cell suspension of 4×10^6 cells/ml solution. This was mixed by slow pipetting and transferred to a sterile syringe. Alginate beads were formed by dripping the solution from the syringe needle (26 gauges) into calcium chloride (102 mM). The beads were allowed to polymerize for 10 min at ambient temperature. After washing twice in physiologic salt, twice in DMEM, respectively, the alginate beads were transferred to 24-well tissue culture dishes with 10 beads per well (Greiner Bio-One, Kremsmuenster, Austria). The cells were cultured in 500 μl per well DMEM supplemented with 10% FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2.5 μg/ml of amphotericin B, and 50 μg/ml of ascorbate-2-phosphate (Sigma).

IMMUNOFLUORESCENCE

After 7, 18, and 25 days of culture, alginate beads were dissolved in alginate-dissolving buffer (55 mM of Na-citrate, 0.15 M NaCl, 30 mM of Na_2EDTA, pH 6.8) and the cells were collected by centrifugation and resuspended in PBS.

Cytospin slides were prepared by cytocentrifugation (500 rpm, 5 min) (Thermo Fisher Scientific Inc., Waltham, MA) and fixed for 30 min with a 4% buffered formaldehyde solution. The slides were blocked in 1% (w/v) casein (Roche Diagnostics, Mannheim, Germany), 0.1% Tween in PBS for 1 h and were then incubated with the primary antibody for 1 h at ambient temperature and subsequently for 16 h at 4°C. Mouse monoclonal antihuman Type VI collagen (MAB3303; Chemicon, Millipore, Billerica, MA) was used at 1:250 dilution. According to the manufacturer, there is no cross-reactivity with Types I, II, III, IV, and V collagen. On a spot-blot we confirmed that the antibody shows no cross-reactivity with Types I and II collagen and that the antibody reacts with goat Type VI collagen.

After three washes with 0.1% Tween in PBS, the slides were incubated with Alexa 488-conjugated antimouse secondary antibody (Molecular Probes, Invitrogen, Carlsbad, CA) at a 1:100 dilution for 1 h. Following three washes, the cells were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). The cells were analyzed using a Leica fluorescent microscope.

DTAF LABELLING

To determine if the native pericellular matrix was remodeled in alginate beads, chondrons were labeled with DTAF (5-([4,6-dichlorotriazin-2-yl]amino)fluorescein hydrochloride) (Sigma), which is a reactive dye that covalently binds to proteins, especially collagens and to polysaccharides [Davison and Galbavy, 1985; Lee and Loeser, 1998]. Chondrons were incubated with 1 mg/ml of DTAF in 0.2 M Na bicarbonate and after 1 min DMEM was added. Subsequently, the chondrons were washed twice with physiological salt and encapsulated in alginate. The DTAF-labeled pericellular matrix of the chondrons was analyzed in the alginate beads using confocal microscopy (Leica Microsystems, Wetzlar, Germany).

REAL-TIME PCR

Alginate beads were dissolved in alginate dissolving buffer (see Immunofluorescence Section) and total RNA was isolated from the cells with the RNeasy Kit (Qiagen, Gaithersburg, MD). DNaseI treatment was performed as described by the manufacturer to remove any contaminating genomic DNA. Total RNA (750 ng) was reverse-transcribed using 250 U/ml of transcriptor reverse transcriptase (Roche Diagnostics), 0.08 U of random primers (Roche diagnostics), and 1 mM of each dNTP (Invitrogen) in transcriptor RT reaction buffer at 42°C for 45 min followed by an inactivation of the enzyme at 80°C for 5 min. Real-time PCR reactions were performed using the SYBR Green Reaction Kit according to the manufacturer's instructions (Roche Diagnostics) in a Light Cycler 480 (Roche Diagnostics). The Light Cycler reactions were prepared in 20 µl total volume with 7 µl of PCR-H_2O, 0.5 µl of forward primer (0.2 µM), 0.5 µl of reverse primer (0.2 µM), 10 µl of Light Cycler Mastermix (LightCycler 480 SYBR Green I Master; Roche Diagnostics), to which 2 µl of five times diluted cDNA was added as PCR template. Primers (Invitrogen) used for real-time PCR are listed in Table I. Specific primers were designed from sequences available in the data banks, based on homology in conserved domains between human, mouse, rat, dog, and cow. The amplified PCR fragment extended over at least one exon border. Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*Ywhaz*), ubiquitin C (*Ubc*), and hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) were used as housekeeping genes and the gene expression levels were normalized for the normalization factor calculated with the equation $\sqrt[3]{(\mathrm{Ywhaz} \times \mathrm{Ubc} \times \mathrm{Hprt1})}$. With the Light Cycler software (version 4), the crossing points were assessed and plotted versus the serial dilution of known concentrations of the standards derived from each gene using Fit Points method. PCR efficiency was calculated by Light Cycler software and the data were used only if the calculated PCR efficiency was between 1.85 and 2.0.

PAPAIN DIGESTION

Alginate beads were digested at 56°C for 18 h in a papain enzyme solution consisting of 5 mM L-cysteine, 50 mM Na_2EDTA, 0.1 M NaAc, pH 5.53 with 3% (v/v) papain (35 U/mg; Worthington; 10 alginate beads in 1 ml of papain enzyme solution).

PROTEOGLYCAN CONTENT

To analyze the proteoglycan content of the alginate beads, the Blyscan assay (Biocolor Ltd, Newtonabbay, Ireland) was performed to determine the sulfated glycosaminoglycans according to the manufacturer's instructions with 40 µl of papain-digested alginate beads. Papain-digested alginate beads were incubated with Blyscan dye reagent for 30 min. After centrifugation, the supernatant was removed and Blyscan Dye Dissociation Reagent was added to the pellet. After 15 min, the absorbance was measured at 656 nm.

DNA ANALYSIS

The total DNA of the alginate beads was quantified using the CyQuant cell proliferation assay kit (Molecular Probes, Eugene, OR) according to the manufacturer's instruction with 50 µl of papain-digested alginate beads. CyQuant GR dye/cell-lysis buffer was added to the papain-digested alginate beads. This was incubated at ambient temperature for 5 min, protected from light. The fluorescence was measured with filters used for fluorescein (~480 nm excitation and ~520 nm emission).

COLLAGEN IMMUNOBLOTTING

After 7, 18, and 25 days of culture, alginate beads were collected and the collagen was extracted as described previously [Chubinskaya et al., 2001]. The beads were dissolved in alginate-dissolving buffer (see Immunofluorescence Section) and the suspension was centrifuged at 100 ***g*** for 10 min at 4°C, resulting in two fractions: the pellet containing the cells with their cell-associated matrix and the supernatant containing the nonbound further removed matrix [Poole et al., 1991]. Both fractions were digested overnight at 4°C with pepsin (Worthington; 100 µg/ml in 0.2 M NaCl, 0.5 M acetic acid). The digests were centrifuged at 12,000 ***g*** for 30 min at 4°C and the supernatants were dialyzed for 48 h at 4°C against 10 mM Tris, 25 mM Na_2EDTA, and lyophilized for 8 h.

The collagen was dissolved in 0.02 M acetic acid and denatured by heating at 95°C for 5 min in NuPage LDS sample buffer (Invitrogen) with NuPage reducing agent (Invitrogen). The collagen was resolved on NuPage 3–8% Tris-Acetate mini gels (Invitrogen). After SDS–PAGE, the proteins were transferred to nitrocellulose membranes (Invitrogen) using the iBlot dry blotting system (Invitrogen) at

TABLE I. Primer Sequences Used for Real-Time PCR

Target gene	Primers	Oligonucleotide sequence	Annealing temperature (°C)	Product size (bp)
Hprt1	Forward	5′-GCTGACCTGCTGGATTACAT-3′	56	260
	Reverse	5′-CTTGCGACCTTGACCATCT-3′		
Ubc	Forward	5′-GCGGTGAACGCCGATGATTAT-3′	56	202
	Reverse	5′-TTTGCCTTGACATTCTCGATGG-3′		
Ywhaz	Forward	5′-GATGAAGCCATTGCTGAACTTG-3′	56	229
	Reverse	5′-CTATTTGTGGGACAGCATGGA-3′		
Acan	Forward	5′-CAACTACCCGGCCATCC-3′	57	160
	Reverse	5′-GATGGCTCTGTAATGGAACAC-3′		
Col1a1	Forward	5′-TCCAACGAGATCGAGATCC-3′	57	191
	Reverse	5′-AAGCCGAATTCCTGGTCT-3′		
Col2a1	Forward	5′-AGGGCCAGGATGTCCGGCA-3′	56	195
	Reverse	5′-GGGTCCCAGGTTCTCCATCT-3′		
Mmp13	Forward	5′-GGAGCATGGCGACTTCTAC-3′	56	208
	Reverse	5′-GAGTGCTCCAGGGTCCTT-3′		

Hprt1, hypoxanthine phosphoribosyltransferase 1; *Ubc*, ubiquitin C; *Ywhaz*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; *Acan*, aggrecan; *Col1a1*, α1(I)procollagen; *Col2a1*, α1(II)procollagen; *Mmp13*, matrix metalloproteinase 13.

program P3 for 8 min after a short wash of the gel in NuPage transfer buffer (Invitrogen).

The membranes were blocked in 2% (w/v) BSA 0.1% Tween in PBS for 1 h and were then incubated with the primary antibody for 2 h. Mouse monoclonal anti-Type II collagen (MAB1330; Chemicon) was used at 1:1,000 dilution. According to the manufacturer, there is no cross-reactivity with Types I, III, V, and IV collagen. On a spot-blot we confirmed that the antibody has no cross-reactivity with Type I collagen and that the antibody reacts with goat Type II collagen. The mouse monoclonal anti-Type I collagen (ab6308; Abcam, Cambridge, UK) was used at 1:1,000 dilution. According to the manufacturer, there is no cross-reactivity with Types II, III, IV, V, VI, VII, IX, X, and XI collagen. We confirmed on a spot-blot that the antibody has no cross-reactivity with Type II collagen and that the antibody reacts with goat Type I collagen.

After three washes with 0.1% Tween in PBS, the membranes were incubated with horseradish peroxidase-conjugated antimouse secondary antibody (DakoCytomation, Glostrup, Denmark) at 1:5,000 dilution for 1 h. Following three washes, immunoreactivity was visualized using Lumi-Light^{plus} (Roche Diagnostics).

ZYMOGRAPHY

Culture medium was replaced with DMEM supplemented with 0.1% BSA 5 h before collecting the medium. Conditioned medium was dissolved in 4× Tris-glycine SDS sample buffer (Invitrogen) and loaded onto a Novex 10% zymogram gel containing 0.1% gelatin (Invitrogen) and separated by SDS–PAGE. The amount of conditioned medium was equalized for the amount of DNA (12.5 μl was applied to the gel). After electrophoresis, the gel was renatured in Novex zymogram renaturing buffer (Invitrogen) at ambient temperature for 30 min with gentle agitation, equilibrated in Novex zymogram developing buffer (Invitrogen) at ambient temperature for 30 min with gentle agitation, and incubated overnight at 37°C in fresh Novex zymogram developing buffer. Transparent bands of gelatinolytic activity were visualized by staining with 0.5% Coomassie Blue R250.

STATISTICAL ANALYSIS

Data are expressed as mean ± SD. Data were statistically analyzed using one-way analysis of variance (ANOVA) or a two-tailed paired *t*-test. Post hoc comparisons were made with Bonferroni correction. The level of significance was set at $P < 0.05$.

RESULTS

PERICELLULAR MATRIX STAINING

To verify whether the chondrocyte and chondron isolations were successful and to investigate the state of the pericellular matrix after 25 days of culture, an immunostaining for Type VI collagen was performed [Poole et al., 1992; Lee et al., 1997]. Just after isolation, the chondrocytes isolated from nucleus pulposus, annulus fibrosus, and articular cartilage showed no staining of Type VI collagen (Fig. 1A–C), whereas in the chondrons this type of collagen was clearly located around the cells (Fig. 1D–F). After 25 days of culture in alginate beads, some Type VI collagen was located around the chondrocytes, but the rim of Type VI collagen around the chondrocytes was always thinner compared with that of the isolated chondrons (Fig. 1G–I). The chondrons showed the same Type VI localization after 25 days of culture in alginate beads as just after isolation (Fig. 1J–L).

Also the DTAF labeling of the collagen in the pericellular matrix of the chondrons showed no differences with time in culture (Fig. 1M–O).

DNA

There were no significant differences found in DNA content between chondrocytes and chondrons at all time points. Neither did the DNA content change over time for the chondrocyte and the chondron populations (data not shown).

LEVEL OF PROTEOGLYCANS

As aggrecan is one of the major proteoglycans in cartilage, its gene expression levels were measured for the different chondron and chondrocyte populations over time. Just after isolation, the highest aggrecan gene expression levels were found for the cells isolated from nucleus pulposus (Fig. 2A, left panel), followed by annulus fibrosus (Fig. 2A, middle panel) and articular cartilage (Fig. 2A, right panel). The aggrecan gene expression levels decreased over time for both chondrocytes and chondrons isolated from the different cartilaginous tissues. The chondrocytes from nucleus pulposus showed higher gene expression levels at day 0 compared with the chondrons, whereas the chondrons showed higher gene expression levels at days 7 and 18. Chondrons isolated from annulus fibrosus had higher gene expression levels at day 0 compared with the chondrocytes, but the chondrocytes showed higher gene expression levels at the other time points. In the cells isolated from articular cartilage the only difference between chondrocytes and chondrons was found at day 0 where the levels in chondrocytes were higher.

The proteoglycan content increased over time for both the chondrocytes and chondrons (Fig. 2B). After 25 days of culture, the alginate beads with nucleus pulposus cells contained the highest amount of proteoglycans (Fig. 2B, left panel), followed by articular cartilage cells (Fig. 2B, right panel). Annulus fibrosus cells produced the lowest amount of proteoglycans (Fig. 2B, mid panel). The chondrons from nucleus pulposus had produced more proteoglycans compared with the chondrocytes after 18 and 25 days of culture. No differences were found in the amount of proteoglycans between chondrocytes and chondrons isolated from annulus fibrosus, whereas chondrons from articular cartilage had produced more proteoglycans after 25 days of culture compared with the chondrocytes.

COLLAGEN ANALYSIS

The Type II collagen gene expression levels increased over time for both chondrocytes and chondrons isolated from annulus fibrosus (Fig. 3A, middle panel) and articular cartilage (Fig. 3A, right panel), whereas the levels in cells from nucleus pulposus were stable over time (Fig. 3A, left panel). The chondrons isolated from nucleus pulposus showed higher Type II collagen gene expression levels at all time points compared with the chondrocytes. A similar effect was seen in the cells from articular cartilage at days 0, 18, and 25. The

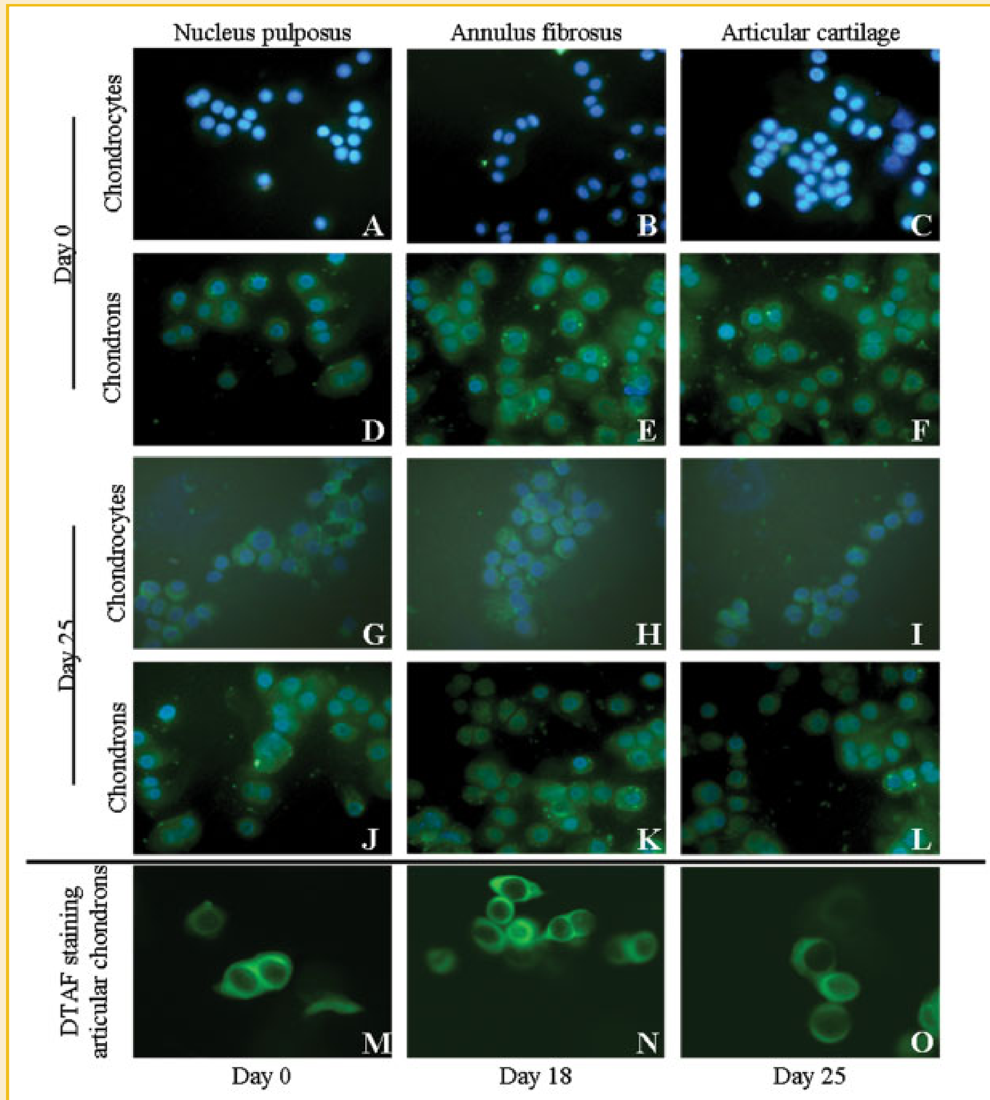

Fig. 1. Staining of the pericellular matrix of chondrocytes and chondrons. A–L: Alexa-488 immunostaining of Type VI collagen (green) and DAPI (blue) staining of chondrocytes and chondrons isolated from nucleus pulposus, annulus fibrosus, and articular cartilage after 0 and 25 days of culture in alginate beads. M–O: 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein hydrochloride staining of the collagen in the pericellular matrix of chondrons from nucleus pulposus, annulus fibrosus, and articular cartilage (shown here) after 0 (M), 18 (N), and 25 (O) days of culture in alginate beads. Magnification, ×400. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

chondrons from annulus fibrosus showed higher gene expression levels compared with the chondrocytes only at days 0 and 7. The chondrons isolated from articular cartilage showed higher Type II collagen gene expression levels after 18 and 25 days of culture compared with the chondrons isolated from the other tissues, whereas the gene expression by the chondrocytes from articular cartilage did not differ from the chondrocytes from the other tissues.

No significant differences were found in Type I gene expression levels for chondrocytes isolated from nucleus pulposus over time and the gene expression levels decreased over time for the chondrons. The chondrocytes from this tissue showed consistently higher gene expression levels compared with the chondrons (Fig. 3B, left panel). For cells isolated from annulus fibrosus Type I collagen gene expression levels increased over time. The only differences between chondrocytes and chondrons were seen at days 18 and 25 where the chondrocytes had higher gene expression levels (Fig. 3B, middle panel). Chondrocytes and chondrons isolated from articular cartilage showed a time-dependent increase in the expression of Type I collagen. The chondrocytes showed higher gene expression levels compared with the chondrons at days 18 and 25 (Fig. 3B, right panel). Just after isolation and after 7 days of culture, Type I collagen gene expression levels by articular cartilage cells were low compared with the cells from the other tissues. After 18 and 25 days, the gene expression levels increased and after 25 days the expression levels of articular cartilage cells were comparable with the gene expression levels by nucleus pulposus chondrocytes (Fig. 3B). After 18 and 25 days, the Type I collagen gene expression levels by annulus fibrosus chondrocytes increased.

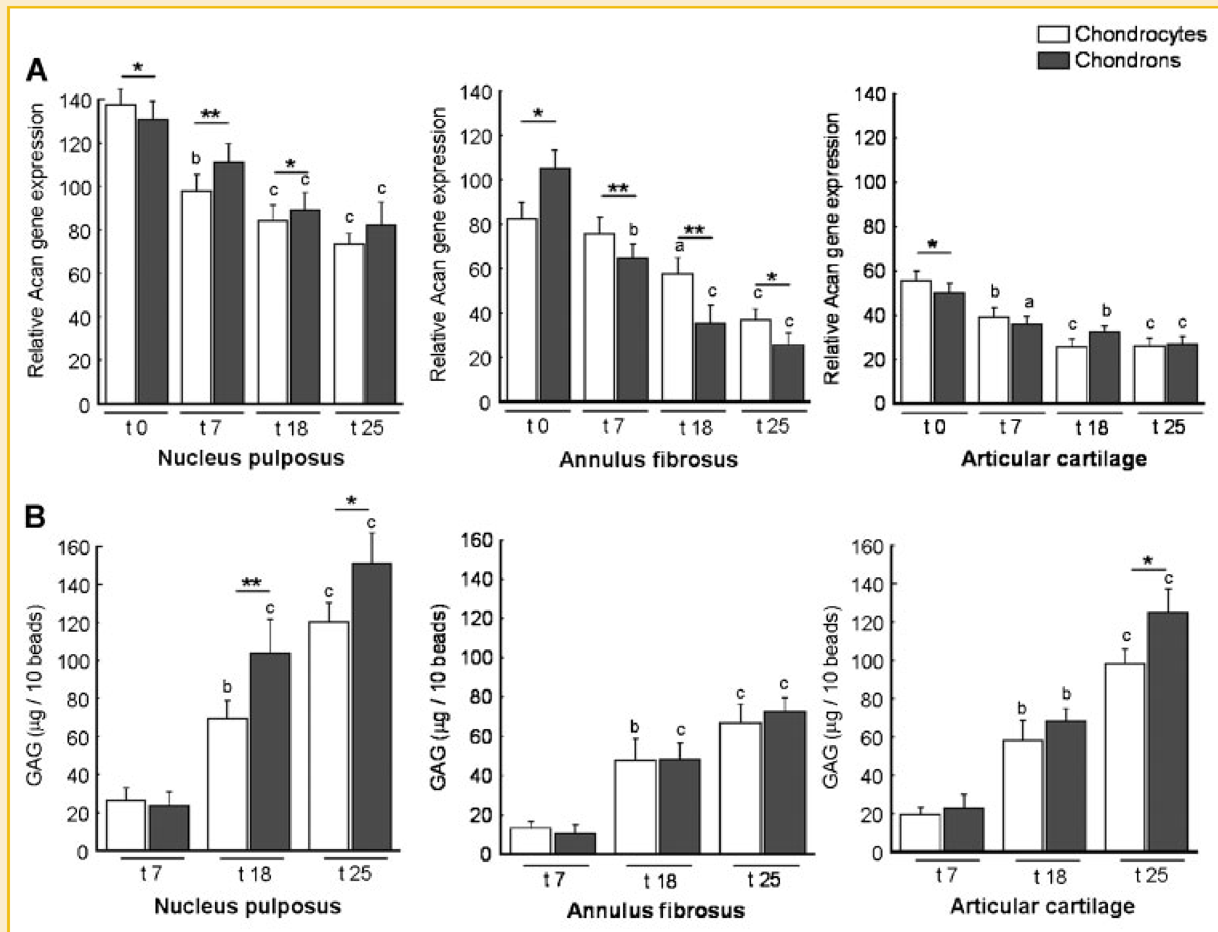

Fig. 2. Aggrecan gene expression and proteoglycan content in cultures of chondrocytes and chondrons in alginate beads. The relative aggrecan (*Acan*) gene expression was measured by real-time PCR (A) and the proteoglycan content was determined as sulfated glycosaminoglycans (B) of alginate beads containing chondrocytes (white bars) and chondrons (grey bars) isolated from nucleus pulposus (left panel), annulus fibrosus (middle panel), and articular cartilage (right panel) after 0, 7, 18, and 25 days of culture. Data are shown as mean ± SD, n = 5. To analyze differences between chondrocytes and chondrons, a two-tailed paired *t*-test was used: $^{*}P<0.05$; $^{**}P<0.01$. To analyze differences between one cell population over time, a one-way analysis of variance (ANOVA) was used. Post hoc comparisons were made with Bonferroni correction. Significant differences compared with T = 0 (A) or T = 7 (B) are indicated: a: $P<0.05$; b: $P<0.01$; c: $P<0.001$.

To analyze collagen on protein level, Western blots were prepared from collagen extracted from the alginate beads containing the cells from nucleus pulposus (Fig. 4A), annulus fibrosus (Fig. 4B), and articular cartilage (Fig. 4C). Type II collagen was deposited in both the cell-associated matrix and the further removed matrix for both chondrocytes and chondrons isolated from the different tissues, but the majority of the Type II collagen was found in the cell-associated matrix. The amount of Type II collagen found in the alginate beads appeared to be similar between chondrocytes and chondrons isolated from one tissue, but only the Type II collagen that was produced by chondrons gave [alpha1(II)]2 and [alpha1(II)]3 chains on Western blot.

In the extracted collagen from the alginate beads, no Type I collagen could be detected on Western blot (Fig. 4D).

METALLOPROTEINASE EXPRESSION

After 7 days of culture in alginate beads, no differences were found in intensity of the Mmp9 and Mmp2 fragments on zymograms between chondrocytes and chondrons and also between the cells isolated from the different tissues (Fig. 5). After 18 and 25 days of culture, more Mmp2 was found in the medium of the chondrocytes isolated from nucleus pulposus compared with the chondrons (Fig. 5A). In conditioned medium of annulus fibrosus cells no differences were found in the levels of Mmp9 and Mmp2 over time and also between chondrocytes and chondrons (Fig. 5B). In conditioned medium of articular cartilage cells, the amount of Mmp2 was increased after 18 and 25 days in both chondrocytes and chondrons compared with day 7. Besides, the active Mmp2 fraction at 69 kD appeared on the zymogram after 18 and 25 days. This fraction was more pronounced in the medium from the chondrocytes compared with that of the chondrons (Fig. 5C). Mmp9 was increased in the medium of articular cartilage chondrocytes after 25 days of culture, but not in the medium of the chondrons.

Culture in alginate beads increased the gene expression levels of *Mmp13* in both chondrocytes and chondrons isolated from

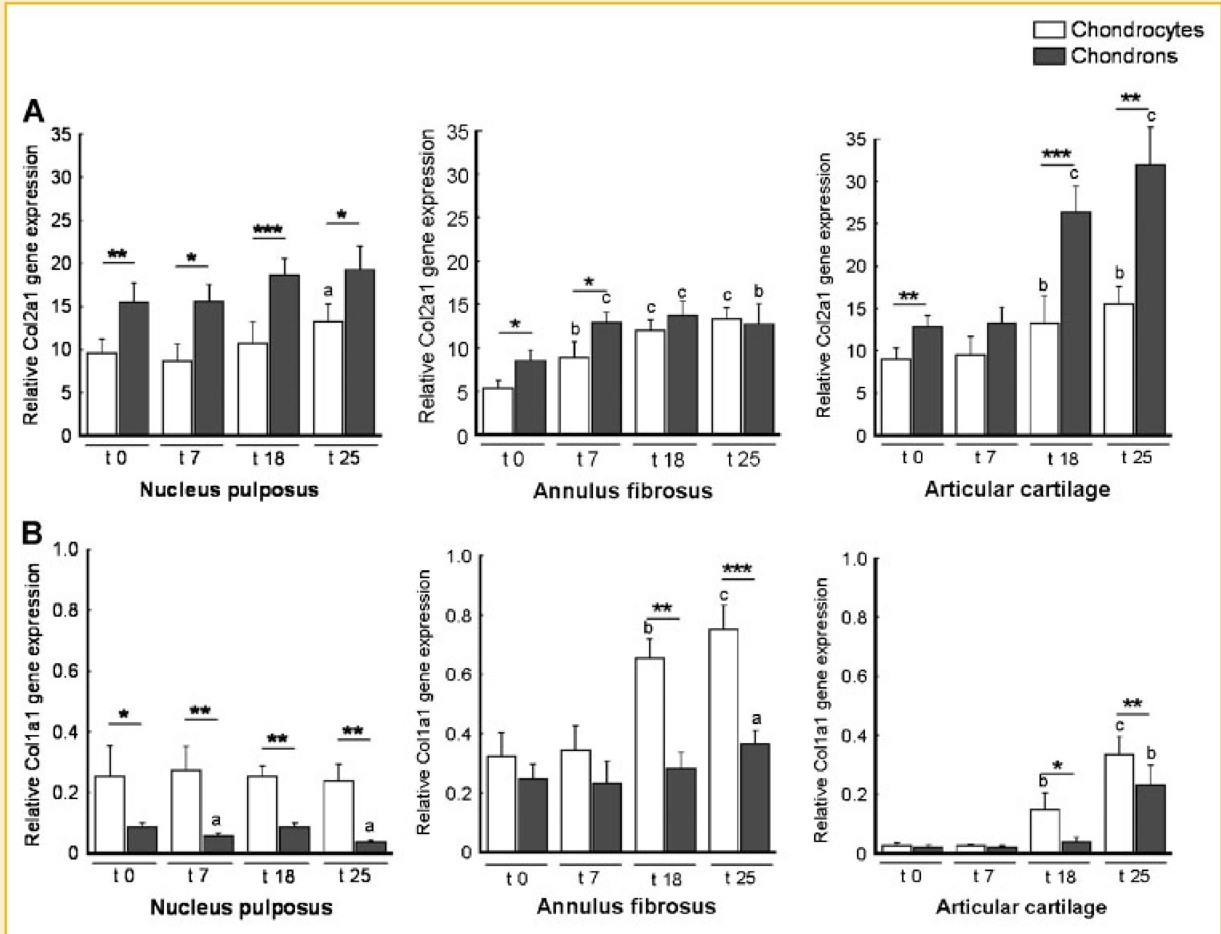

Fig. 3. Gene expression levels of Types II and I collagen in cultures of chondrocytes and chondrons in alginate beads. Real-time PCR was performed on reverse-transcribed RNA isolated from chondrocytes (white bars) and chondrons (grey bars) derived from nucleus pulposus (left panel), annulus fibrosus (middle panel), and articular cartilage (right panel) after 0, 7, 18, and 25 days of culture. The results are presented as relative gene expression of Type II collagen (Col2a1, A) and Type I collagen (Col1a1, B). Data are shown as mean $\pm$ SD, n = 5. To analyze differences between chondrocytes and chondrons, a two-tailed paired *t*-test was used: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. To analyze differences between one cell population over time, a one-way analysis of variance (ANOVA) was used. Post hoc comparisons were made with Bonferroni correction. Significant differences compared with T = 0 are indicated: a: $P < 0.05$; b: $P < 0.01$; c: $P < 0.001$.

the different cartilaginous tissues over time, but the increase by the chondrocytes was much more pronounced (Fig. 6). Just after isolation no differences were found in *Mmp13* gene expression levels between chondrocytes and chondrons and also not between these cells from the different tissues. However, after 7 days, the gene expression levels of *Mmp13* by all the chondrocytes started to increase (about fivefold). After 18 days the *Mmp13* gene expression levels were increased 10-fold by chondrocytes from articular cartilage and about 35-fold by chondrocytes from nucleus pulposus and annulus fibrosus compared with immediately after isolation. After 25 days they were increased 60-fold by articular cartilage chondrocytes and 165-fold by the cells from annulus fibrosus and nucleus pulposus. Compared with the chondrocytes the increase of the *Mmp13* gene expression level was relatively small by the chondrons after 25 days (6-fold by articular cartilage chondrons and 20-fold by chondrons from annulus fibrosus and nucleus pulposus).

DISCUSSION

Previous studies that have compared matrix production by chondrocytes and chondrons were performed in pellet cultures to maintain the chondrocytic phenotype [Larson et al., 2002; Graff et al., 2003]. However, in pellet culture, the chondrocytes tend to exit their pericellular matrix, leaving it behind as an empty shell. The superficial rim of these pellets was more fibrocartilaginous in nature than hyaline cartilage with abundant Type I collagen and decreased Type II collagen and proteoglycan content [Lee and Loeser, 1998; Larson et al., 2002; Graff et al., 2003]. In this study, the chondrons were cultured in alginate beads for up to 25 days and Type VI collagen staining did not differ from the staining just after isolation. Also the DTAF, which is a reactive dye that covalently binds to proteins, especially collagens and polysaccharides [Davison and Galbavy, 1985; Lee and Loeser, 1998] labeling of the pericellular matrix had the same appearance over time, suggesting that the

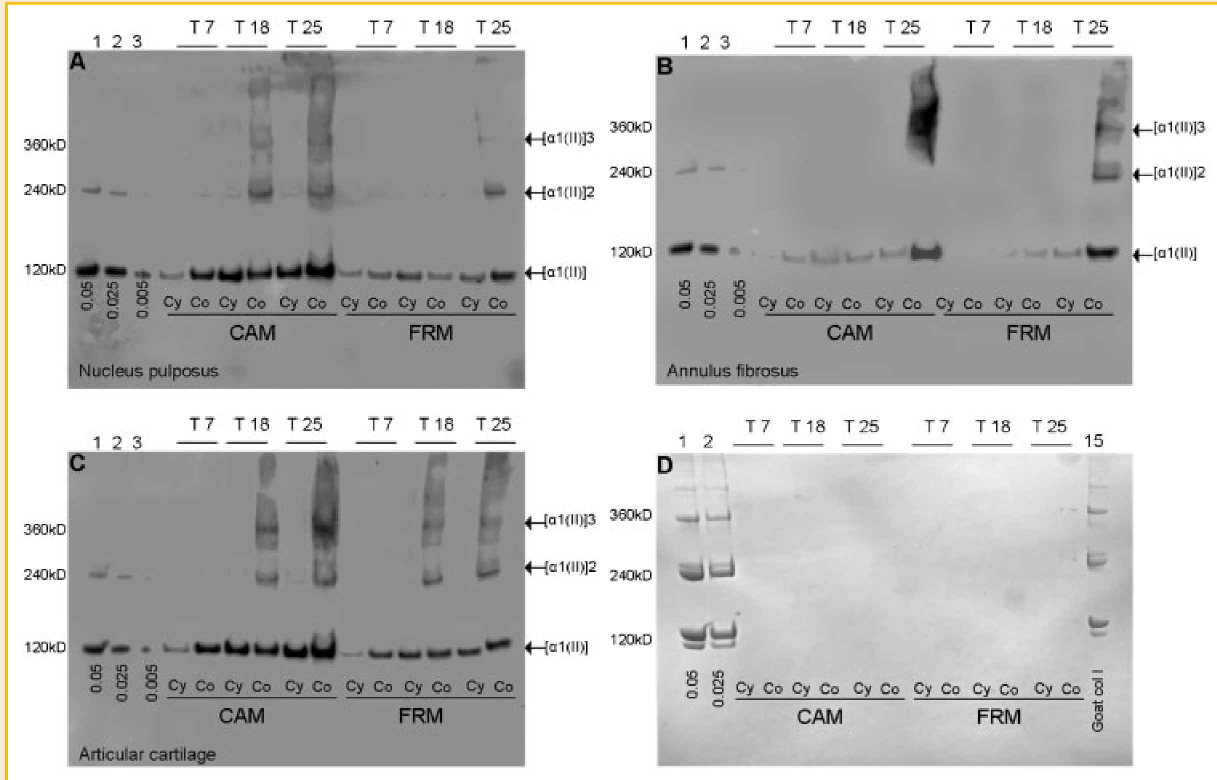

Fig. 4. Western blots of collagen extracted from alginate beads containing chondrocytes or chondrons. Immunoblot of Type II collagen extracted from the cell-associated matrix (CAM) and further removed matrix (FRM) of alginate beads cultured for 7, 18, and 25 days containing chondrocytes (Cy) and chondrons (Co) isolated from nucleus pulposus (A), annulus fibrosus (B), and articular cartilage (C). Immunoblot of Type I collagen (D) revealed no fragments in the collagen extracted from alginate beads with cells isolated from annulus fibrosus (shown here), nucleus pulposus, or articular cartilage. Lane 15 of the Type I collagen Western blot contains Type I collagen isolated from a goat meniscus (0.02 μg). The collagen fragments are indicated by arrows; the separate [α(I)II] chains have a molecular mass of approximately 120 kD, two chains have a mass of 240 kD, and three chains of 360 kD. The first lanes contain standards of 0.05, 0.025, and 0.005 μg of Type II collagen (Fig. 4A–C, lanes 1, 2, and 3) or 0.025 and 0.005 μg of Type I collagen (Fig. 4D, lanes 1 and 2).

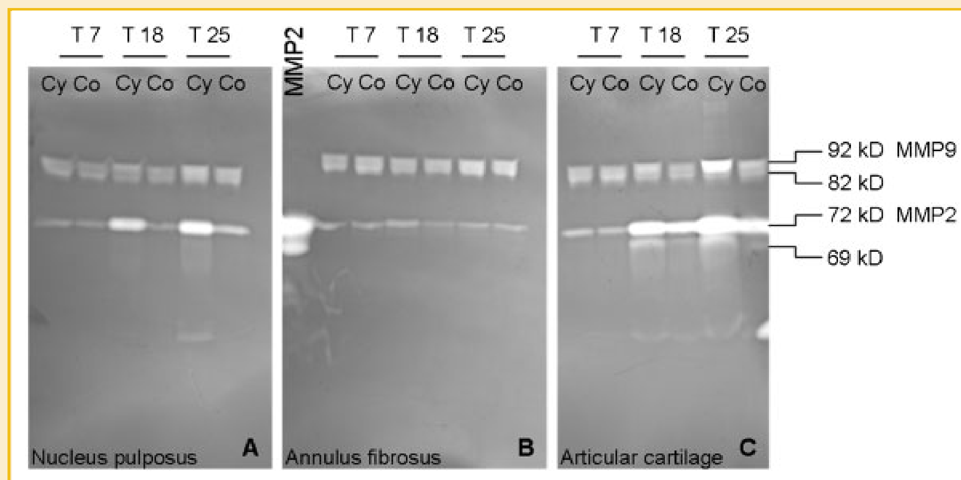

Fig. 5. Zymography of gelatinases in conditioned medium of alginate beads containing chondrocytes or chondrons. Conditioned medium of alginate beads cultured for 7, 18, and 25 days containing chondrocytes (Cy) and chondrons (Co) isolated from nucleus pulposus (A), annulus fibrosus (B), and articular cartilage (C) was analyzed for matrix metalloproteinase 2 (Mmp2) and metalloproteinase 9 (Mmp9). The first lane of Figure 5B contains a recombinant Mmp2 standard.

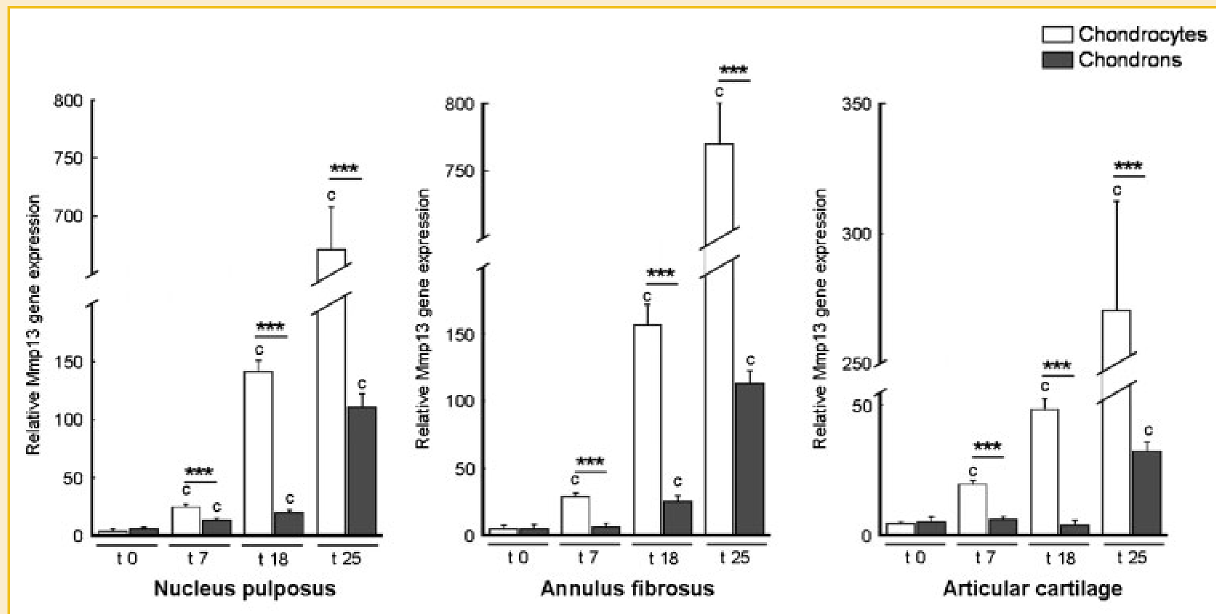

Fig. 6. Gene expression levels of metalloproteinase 13 (*Mmp13*) by cultured chondrocytes and chondrons. Real-time PCR was performed on reverse-transcribed RNA isolated from chondrocytes (white bars) and chondrons (grey bars) derived from nucleus pulposus (left panel), annulus fibrosus (middle panel), and articular cartilage (right panel) after 0, 7, 18, and 25 days of culture. The results are presented as relative gene expression of *Mmp13*. Data are shown as mean ± SD, n = 5. To analyze differences between chondrocytes and chondrons, a two-tailed paired *t*-test was used: $^{***}P < 0.001$. To analyze differences between one cell population over time, a one-way analysis of variance (ANOVA) was used. Post hoc comparisons were made with Bonferroni correction. Significant differences compared with T = 0 are indicated: c: $P < 0.001$.

native pericellular matrix was not degraded during culturing of these cells in alginate beads. Also no empty chondron shells were found, thus indicating that the cells do not leave their environment if cultured in alginate. So encapsulation of chondrons in alginate is a good method to preserve most if not all of the ECM components present in the pericellular matrix.

After 25 days of culture in alginate beads, the chondrocytes have produced Type VI collagen that is located pericellular, but the rim of Type VI collagen around the chondrocytes is thinner compared with that of the chondrons. Others have also described that chondrocytes form a pericellular matrix in alginate beads [Guo et al., 1989; Lee and Caterson, 1995; Hsieh-Bonassera et al., 2009]. So, the chondrocytic phenotype is maintained and a thin rim of Type VI collagen is produced pericellularly by the chondrocytes during a culture period of 25 days in alginate beads, but it might take more time before the full thickness of a native pericellular matrix is reached.

The amount of sulfated glycosaminoglycans that was found in the alginate beads after a culture period of 25 days was significantly higher in chondrons isolated from nucleus pulposus and articular cartilage compared with the chondrocytes. This is in line with the alleged positive effect that the pericellular matrix has on matrix metabolism by chondrocytes [Larson et al., 2002; Graff et al., 2003]. The differences in the amount of sulfated glycosaminoglycans, not only between chondrons and chondrocytes, but also between the cells from the different tissues, were found at days 18 and 25, but not yet at day 7. This might be an effect of the isolation; by releasing the cells from the ECM, they are stimulated to produce a new one. Once they have produced sufficient amounts of proteoglycans, the cartilage-specific effects and the effect of the pericellular matrix become apparent.

The gene expression levels of aggrecan do not seem to correlate with the amount of proteoglycans found in the alginate beads. However, considering that in this assay other proteoglycans, such as biglycan and decorin, which were probably also produced by the cells, are also measured, it is not possible to compare these directly. The gene expression levels of aggrecan are different among the cells isolated from the various cartilaginous sources, especially at the onset of the cultures. Over time the gene expression of aggrecan decreases, possibly to a basic level required for just maintaining aggrecan in the alginate beads. This supports our suggestion put forward in the former paragraph that the cells first produce a sufficient amount of proteoglycans for the ECM and afterwards the proteoglycans are remodeled and maintained at a level comparable with the native tissues.

The higher Type II collagen and the lower Type I collagen gene expression levels found in the chondrons indicates that the chondrons form a more cartilage-like ECM than the chondrocytes. This strongly suggests that the pericellular matrix preserves the maintenance of the differentiated chondrocyte phenotype. This is in line with the results found in other studies [Lee and Loeser, 1998; Larson et al., 2002, Graff et al., 2003].

A previous study reported that when cultured in pellets, chondrons produced more Type II collagen compared with chondrocytes [Larson et al., 2002]. Whether this also occurred in our cultures is difficult to conclude as we have used goat cells, making it very difficult to get a quantitative measurement. Although our immunoblot analysis is not quantitative, the advantage is that

the molecular weights of the proteins can be visualized. This revealed that the Type II collagen extracted from the alginate beads with chondrons was cross-linked. [alpha1(II)]2 and [alpha1(II)]3 chains were present next to the alpha1(II) chains. The cross-links that are present in the [alpha1(II)]2 and [alpha1(II)]3 chains cannot be broken by denaturing conditions such as heating in the presence of a reducing agent. These cross-links were absent in Type II collagen produced by chondrocytes. Lysyl oxidase and lysyl oxidase-like enzymes catalyze the formation of aldehydes from lysine and hydroxylysine residues in the telopeptides. Subsequent spontaneous reactions result in the formation of intermediate cross-links which are later converted into mature cross-links [Csiszar, 2001; Hayashi et al., 2004]. This suggests that the pericellular matrix modulates either the expression or the activity of lysyl oxidase and lysyl oxidase-like enzymes. As the mature cartilaginous tissues also contain the [alpha1(II)]2 and [alpha1(II)]3 chains, the collagen network that is produced by the chondrons does resemble more the collagen network found in the native tissues.

We observed a discrepancy between relative gene expression levels of Type I collagen and the immunoblot analysis of this collagen. We were able to quantify Type I collagen gene expression by all the cells, but we were not able to find any Type I collagen in the collagen extracted from the alginate beads. This suggests that the Type I collagen mRNA that was found in the cells was either not translated into protein, or too little was translated into protein for its detection by Western blot, or the Type I collagen was degraded intracellularly.

Not only the anabolic activities were influenced by maintaining the pericellular matrix, but differences were also found in the expression of catabolic factors. The amount of Mmp2 was increased by the chondrocytes from nucleus pulposus and articular cartilage compared with the chondrons. Besides the increase in the level of Mmp2, activated Mmp2 and increased levels of Mmp9 were found in the medium of articular chondrocytes. It is likely that the altered cell–ECM interaction caused by removal of the native pericellular matrix plays a role in the increased expression of the gelatinases [Kerkvliet et al., 2003]. Fibronectin could be a candidate for this process as it is a component of the cartilage ECM, and it is known that fibronectin fragments can stimulate the release of several MMPs including MMP9 and MMP2 [Bonaldo and Colombatti, 1989; Chu et al., 1990; Salter et al., 1992; Loeser, 1993; Homandberg and Wen, 1998; Peters et al., 2002]. Although fibronectin is also a component of the native pericellular matrix, the removal of Type VI collagen might have altered the interactions of the chondrocyte receptors with fibronectin causing an increased expression of *Mmp2* and *Mmp9*. That no differences were found in the expression of *Mmp2* and *Mmp9* between chondrocytes and chondrons from annulus fibrosus appears to be in line with a study in which chondrocytes from annulus fibrosus proved to be unresponsive to fibronectin fragments [Aota et al., 2005]. It could be that annulus fibrosus cells do not respond to fibronectin or that the expression of integrin receptor $\alpha5\beta1$ is different in annulus fibrosus cells compared with the chondrocytes from nucleus pulposus and articular cartilage; this intriguing difference between the different cells remains to be investigated.

The increased *Mmp13* gene expression by the chondrocytes is probably also a consequence of changes in the cell–ECM intractions. Not only fibronectin (fragments) [Forsyth et al., 2002; Stanton et al., 2002; Loeser et al., 2003], but also fibrillar Types I and II collagen can induce *Mmp13* [Ronziere et al., 2005; Xu et al., 2005; Galois et al., 2006; Freyria et al., 2009]. The chondrocytes probably came into direct contact with fibrillar collagen in the ECM, which was prevented by the pericellular matrix of the chondrons. These fibrillar collagens could interact with collagen receptors such as the discoidin domain 2 (DDR2) receptor and integrins. DDR2 is associated with osteoarthritis [Poole, 1997; Vogel et al., 2006; Sunk et al., 2007; Xu et al., 2007] and silencing DDR2 in the C-28/I2 chondrocyte cell line significantly reduced collagen-induced *MMP13* upregulation [Xu et al., 2005]. Blocking antibodies against $\alpha1$ and $\beta1$ integrin subunits also inhibited collagen-dependent induction of *MMP13* by MC615 chondrocytes [Ronziere et al., 2005]. Based on gene expression levels, no correlation could be found between collagen receptors (integrin and DDR) and a collagen-induced *MMP13* upregulation [Galois et al., 2006; Freyria et al., 2009]. Subsequent functional receptor studies are needed to further unravel the role of receptor-based collagen-induced *MMP13* upregulation by primary chondrocytes.

Although this and other studies have shown that chondrons have a greater potential for cartilage tissue engineering than chondrocytes [Lee and Loeser, 1998; Larson et al., 2002; Graff et al., 2003], the number of cells that can be obtained remains a problem and expansion of both chondrons and chondrocytes leads to dedifferentiation [Bonaventure et al., 1994; Lee and Loeser, 1998; Stewart et al., 2000]. Dedifferentiated chondrocytes can redifferentiate in the direction of a chondrocyte [Bonaventure et al., 1994], but a new native-like pericellular matrix is not automatically formed. It is known that scaffold properties, age of the chondrocytes, and culture conditions influence the production of a native-like pericellular matrix [Chang et al., 1997; Fraser et al., 2006; DiMicco et al., 2007; Hsieh-Bonassera et al., 2009], but the mechanisms behind this are still unknown. As the next generation of therapy for cartilage defects will probably consist of a autologous chondrocyte implantation (ACI) with cells cultured within a three-dimensional matrix [Temenoff and Mikos, 2000; Marlovits et al., 2006; Wakitani et al., 2008] and the native pericellular matrix improves cartilage formation, it might be worth to search for a scaffold which promotes the formation of or resembles a pericellular matrix.

This study has demonstrated that maintaining the native chondrocyte's pericellular matrix affects both anabolic and catabolic activities. Although the different chondrocyte populations maintain their chondrocytic phenotype in alginate beads, the presence of the native pericellular matrix seems to preserve the cartilaginous phenotype even more. This suggests that the ECM surrounding the chondrocytes is essential for maintaining the proper composition of the interchondron ECM and that preserving the thin matrix layer surrounding the chondrocytes improves cell-induced cartilage formation.

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