Alterations in CD44 isoforms and HAS expression in human articular chondrocytes during the de- and re-differentiation processes

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Abstract. The purpose of this study was to investigate the expression of different CD44 and hyaluronan synthase isoforms in cartilage, their alterations during the chondrocyte dedifferentiation process in monolayer culture and during the redifferentiation process on 3D scaffolds. Chondrocytes isolated from human articular cartilage were cultured as a monolayer for up to 36 days and were seeded on two different 3D scaffolds (HYAFF® 11 and Bio-Gide®). Expression levels of *CD44s, CD44-lt, CD44-st, HAS1, HAS2, HAS3* and *UDPGD* were determined by real-time RT-PCR at different time points. At the protein level CD44 and CD90 were analyzed by flow cytometry. *HAS2* was found to be the predominantly expressed hyaluronan synthase in chondrocytes and was not subjected to any regulation during the dedifferentiation process. *CD44s, CD44-lt, CD44-st* and *UDPGD*, however, were upregulated immediately after cell isolation. In addition, a high cell density was found to significantly increase *CD44-st* and *CD44-lt* expression. Redifferentiation on 3D scaffolds reversed the increase of the CD44 expression. Our data point out that CD44 expression does not correlate with matrix assembly in chondrocytes and that CD44 has a regulatory function in chondrocytes, not necessarily on differentiation, but probably on proliferation.

Introduction

Cartilage is an avascular, aneural tissue consisting of a single cell type, the chondrocyte. The main function of the chondrocytes is to maintain the metabolism of the complex extracellular matrix (ECM) via synthesis and degradation of the matrix components. In addition to the collagens (type II, IX, XI), the proteoglycans, particularly aggrecan, comprise the most important components of the ECM. The glycosaminoglycan, hyaluronan, is also present throughout the cartilage, but at substantially lower concentrations (1). Nevertheless, this small amount contributes significantly to the organisation and integrity of the ECM. Often, a multiplicity of aggrecan molecules binds to a single hyaluronan filament, forming high molecular aggregates (2-4). These aggregates are, in turn, anchored to the surface of chondrocytes by CD44, the principal hyaluronan receptor (5-7). Thus, CD44 plays a critical part in the retention of hyaluronan-proteoglycan aggregates to the chondrocyte cell surface (8). However, also other functions of CD44 in many different cell types including cell aggregation, cell migration and proliferation were described (9).

CD44 was the first identified transmembrane hyaluronan-binding protein (9). It is a polymorphic transmembrane glycoprotein (10) and consists of four functional domains: the distal extracellular domain, which acts as the principal hyaluronan binding site; the proximal extracellular domain; the transmembrane domain; and the cytoplasmic domain, which has been shown to interact with cytoskeletal proteins (11). The *CD44* gene is composed of 20 exons with two principal sites of alternative splicing (4). The standard form, *CD44s*, is encoded by exons 1-5, 16-18, and 20. Exons 6-15 (also known as variant exons 1-10) are variably expressed. Alternative splicing in this region gives rise to several transcript variants. Although the distinct physiologic relevance of these isoforms is not clear, some appear to play an important role in tumor cell metastasis (4,12). Another site of alternative splicing concerns exons 19-20 and influences the length of the cytoplasmic domain. Most common isoforms, including *CD44s*, express exon 20 resulting in a 70 amino-acid-long cytoplasmic domain *(CD44-lt)*. Utilization of exon 19 rather than exon 20 leads to a 3 amino-acid-long, 'short tail' intracellular domain *(CD44-st)* that lacks the capacity for cytoskeletal interactions (4,11). It has been shown that chondrocytes express *CD44-lt*, but in previous studies, *CD44-st* expression has also been demonstrated (4,8,13).

More than 20 years ago, Goldberg and Toole reported that chondrocytes undergoing a dedifferentiation process show a reduced capacity for matrix assembly (14). Previous

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studies that analyzed the dedifferentiation process were focused on the regulation of matrix components, such as aggrecan and collagens (15,16). However, nothing is known about the expression of CD44. To better understand which role CD44 plays during the dedifferentiation and re-differentiation processes, we analyzed *CD44* isoform expression in human articular cartilage and chondrocytes in monolayer culture or rather on 3D scaffolds. Since hyaluronan synthases (HAS) and UDP-glucose dehydrogenase (UDPGD) have an essential influence on hyaluronan synthesis, and thus, on matrix assembly, we also analyzed the expression of all *HAS* and *UDPGD*.

Materials and methods

Isolation and monolayer culture of human articular chondrocytes. Human articular cartilage samples were collected from the hips of ten patients (aged 15-88 years) with no history of joint disease, who were scheduled to undergo joint replacement following femoral neck fracture (approved by the Ethics Board of the University of Vienna; code: 184/98). Non-calcified cartilage was dissected from the bone immediately after surgery. The cells were isolated and cultivated in monolayer culture according to our standard operation procedure, as previously described (15). Chondrocytes from three different donors were pooled after cell isolation for dedifferentiation experiments. From the same cartilage used for the cell culture a fraction was pooled and used as native cartilage control. Cells isolated from the ligamentum capitis femoris were used as a fibroblast control. For cell density experiments, the cells from four donors (4 independent experiments) were seeded on 24-well plates at the following cell densities for 48 h: $5x10^3$, $2.5x10^4$, $1x10^5$ and $5x10^5$ cells per cm^2. Medium was changed once after 24 h. Chondrocytes from three donors were pooled, cultivated for 6 weeks in monolayer culture and were then seeded on two scaffolds ($1x10^6$ cells/cm^2) consisting of hyaluronan (Hyaff-11®, Fidia, FAB, Italy) or porcine collagen I/III (Bio-Gide®, Geistlich Pharma, Wolhusen, Switzerland). Culture medium for both, monolayer culture and 3D culture consisted of DMEM, containing 10% fetal calf serum, 2 g/l HEPES, 1% L-glutamine, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone (all from Life Technologies), and 50 mg/l ascorbic acid (Sigma-Aldrich, St. Louis, MO).

RNA extraction and purification. Total RNA was obtained from cells being cultivated in monolayer culture and on 3D scaffolds. Cells were harvested after cell isolation (day zero), at day one, day two, and then every second day, by adding 1 ml of TRI Reagent™ (Sigma-Aldrich). Lysis of the cells was performed directly on the culture dish. RNA isolation was performed according the standard protocol.

For total RNA extraction from native cartilage, slices of human articular cartilage were frozen in liquid nitrogen and ground using a mortar and pestle. Further steps were performed using the RNeasy® plant mini kit (Qiagen, Germany). The procedure for isolating total RNA was executed as described in the RNeasy mini handbook (Qiagen, 06/2001).

cDNA synthesis. Total RNA (0.1-1 μg) was diluted with nuclease-free water to a volume of 15 and 4 μl iScript™ reaction mix, as well as 1 μl iScript reverse transcriptase were added (Bio-Rad Laboratories, CA, USA). The reaction mixture was incubated for 5 min at 25˚C, for 30 min at 40˚C, and for 5 min at 85˚C.

Primers and probes for quantitative analyses. Primers and probes were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), which generates oligonucleotides with similar melting temperatures and minimal self complementarity. To avoid amplification of genomic DNA, the probes were placed at the junction of two exons. Gene specificity of the primers and probes and the absence of DNA polymorphism were confirmed by BLAST searches. Primers and probes were synthesised by biomers.net (Ulm, Germany). Primer concentrations were tested for each primer at concentrations of 50, 300, and 900 nM, choosing the combination that displayed the lowest Ct value. Primer sequences are shown in Table I.

RT-PCR. An advantage cDNA PCR kit (Clontech, CA, USA) was used to perform RT-PCR. The cycle parameters proposed by the manufacturer were changed as follows: 94˚C for 1 min; 35 x (95˚C for 15 sec, 68˚C for 90 sec); and 68˚C for 3 min. Primer sequences: CD44v forward primer: 5'-ata-ttg-ctt-caa-tgc-ttc-agc-tcc-ac-3'; CD44v reverse primer: 5'-ccc-atg-tga-gtg-tcc-atc-tga-ttc-3'. The amplified products were analyzed by electrophoresis on 1.8% agarose gel followed by staining with ethidium bromide.

Real-time PCR amplification and analysis. Real-time PCR amplification was performed and monitored using an ABI Prism® 5700 sequence detection system (Perkin-Elmer Applied Biosystems, CA, USA). The master mix was based on the Brilliant™ QPCR Master mix (Stratagene, CA, USA). The thermal cycling conditions comprised the initial steps at 50˚C for 2 min and at 95˚C for 10 min. Amplification of the cDNA products was performed with 40 PCR cycles, consisting of a denaturation step at 95˚C for 15 sec and an extension step at 60˚C for 1 min. For SYBR Green amplification, an additional dissociation step (95˚C for 15 sec, 60˚C for 20 min, and 95˚C for 15 sec) was performed. 18S rRNA was chosen as the internal standard, using the predeveloped Taq Man® assay (Applied Biosystems). All cDNA samples (3 μl in 25 μl) were analyzed in triplicate. The final numeric value was calculated as the ratio of the gene to 18S rRNA and was expressed in arbitrary units.

FACS. Chondrocytes from monolayer culture were harvested by incubation with 2 mM EDTA for 15 min. Cell count and viability were determined with a Casy cell counter (Schärfe System, Reutlingen, Germany). Viable cells ($2.5x10^5$) were resuspended in FACS buffer (PBS containing 1% fetal calf serum and 0.1% sodium azide) and washed once by centrifugation for 4 min at 1,250 rpm. After resuspension in 100 μl FACS buffer, the cells were incubated with anti-CD44 APC-conjugated (BD Bioscience, NJ, USA) or anti-CD90 FITC-conjugated (BD Bioscience) antibodies or isotype controls for 30 min at room temperature in the dark. Subsequently, the cells were washed twice with FACS buffer and

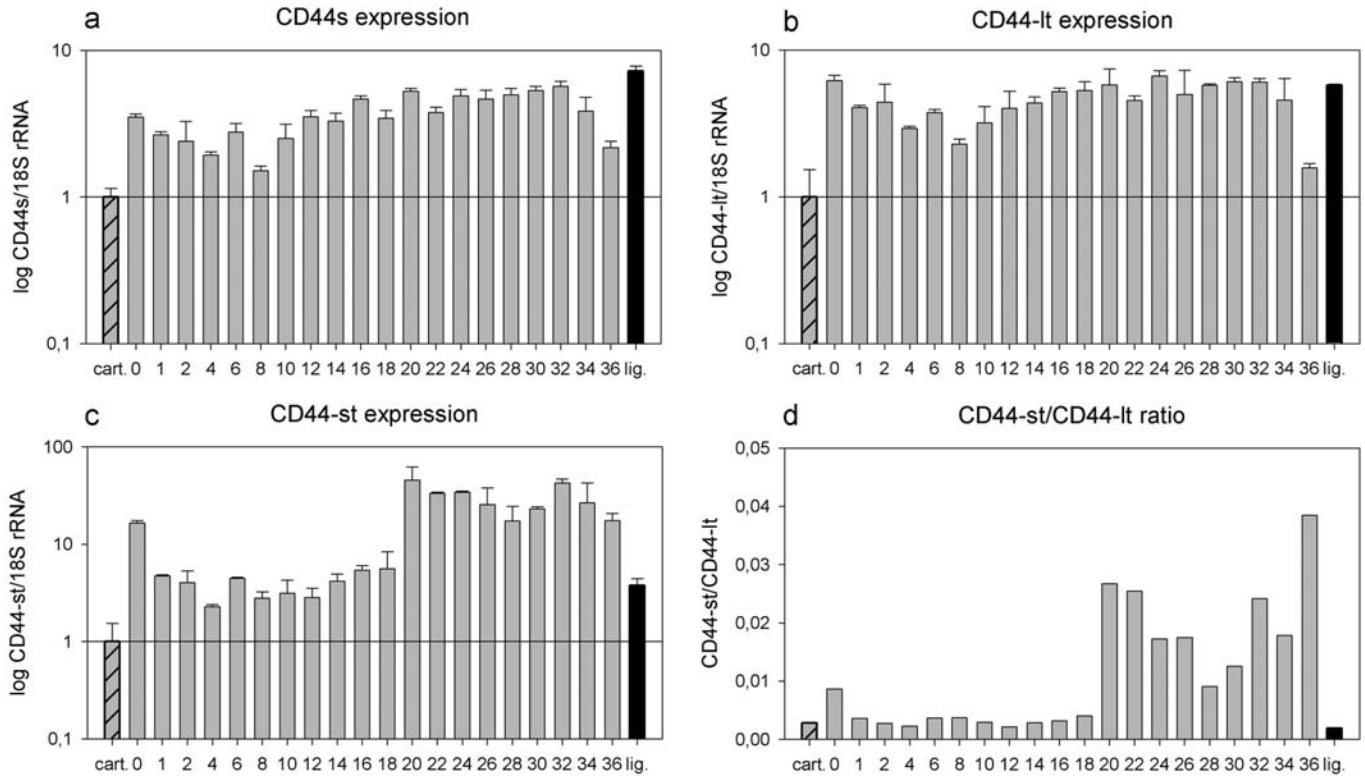

Figure 1. Real-time PCR analysis of *CD44* in native cartilage (cart.), chondrocytes in monolayer culture (day 0-36) and ligament fibroblasts (lig.). Gene expression was normalized to *18S rRNA* expression. Data were log transformed and expressed as mean ± SD of real-time PCR triplicate analysis.

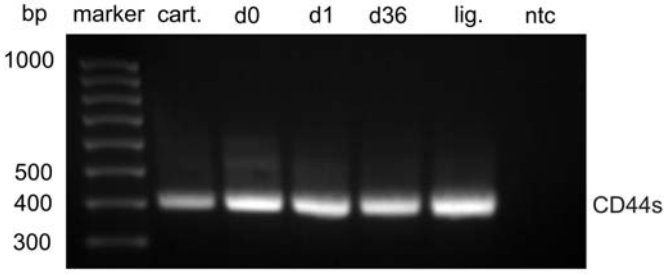

Figure 2. mRNA expression of *CD44* by RT-PCR. Native cartilage (cart.), chondrocytes in monolayer culture at different time points (days 0, 1 and 36), and ligament fibroblasts (lig.) were analyzed for *CD44* isoform expression.

analyzed by flow cytometer (Cytomics FC 500, Beckman Coulter, CA, USA) with CXP software.

Statistical analysis. All samples were assayed by real-time PCR in triplicates. The values were log transformed and reported as the mean ± SD of the real-time PCR analyses. For analysis of significance the Mann-Whitney U test of SPSS 11.5 was applied.

Results

CD44 isoforms expression by real-time PCR and RT-PCR. In order to detect only the standard form of *CD44*, a probe across the exon 5 and 16 boundary, excluding the variable region, was designed. Real-time PCR showed an increase (3.5-fold) in *CD44s* expression immediately after cell isolation (day zero) compared to native cartilage (Fig. 1a). During monolayer culture, *CD44s* expression fluctuated, but always remained at a higher level than native cartilage (1.5-5-fold). In ligament fibroblasts *CD44s* expression was also high (7-fold) compared to native cartilage. Primers flanking the variable exons 6-15 were used to detect alternative splicing variants within the extracellular domain of CD44. In all samples only one intense band at 400 bp, the expected size for CD44s, as well as a slight smear of higher molecular weight was present (Fig. 2). Attempts to amplify other isoforms out of this smear by RT-PCR resulted always in the 400 bp product again (data not shown).

CD44 isoforms arising from the second alternative splicing site (exons 19-20) and affecting the intracellular domain of CD44 were analyzed by a forward primer across the exon 17 and 18 boundary and a reverse primer either on exon 19 *(CD44-st)* or exon 20 *(CD44-lt)*. *CD44-lt* expression showed a time course very similar to *CD44s*. From day zero, *CD44-lt* expression ranged between 1.5- and 6-fold that of native cartilage (Fig. 1b). *CD44-st* expression, however, dramatically increased in the monolayer culture (Fig. 1c). During the first three weeks in the monolayer culture, *CD44-st* expression was, on average, 5-fold higher than that in native cartilage. After about three weeks, expression increased up to 42-fold compared to native cartilage. In contrast, ligament fibroblasts expressed *CD44-st* similar to chondrocytes during the first three weeks of culture. Since absolute quantification of *CD44-st* was not possible with real-time PCR, the ratio between *CD44-st* and *CD44-lt* was calculated. In native cartilage, ligament fibroblasts, and chondrocytes during the first three weeks culture time, *CD44-st* expression represented

Table I. Description of the designed primers and probes.

mRNA template		Primer sequence
CD44s	Left:	5'-ATCACCGACAGCACAGACAG-3'
	Right:	5'-CCATGTGAGTGTCCATCTGATT-3'
	Probe:	5'-ATCCCTGCTACCAGAGACCAAGACAC-3'
CD44-lt	Left:	5'-TCCAGAATGGCTGATCATCTT-3'
	Rght:	5'-TGAGTCCACTTGGCTTTCTGT-3'
CD44-st	Left:	5'-TCCAGAATGGCTGATCATCTT-3'
	Right:	5'-TCATAGACCTCCTAACAGCAAGAA-3'
CD44v	Left:	5'-ATATTGCTTCAATGCTTCAGCTCCAC-3'
	Right:	5'-CCCATGTGAGTGTCCATCTGATTC-3'
HAS1	Left:	5'-TGTCAGAGCTACTTCCACTGTGTAT-3'
	Right:	5'-GTACCCAGGAACTTCTGGTTGTA-3'
	Probe:	5'-CAGCGGTCCTCTAGGCCTATATAGGAA-3'
HAS2	Left:	5'-GAGGACGACTTTATGACCAAGAG-3'
	Right:	5'-AAAGAGTGTGGTTCCAATTATTCTC-3'
	Probe:	5'-CTGAACAAGATGCATTGTGAGAGGTTT-3'
HAS3	Left:	5'-GCGATTCGGTGGACTACAT-3'
	Right:	5'-GGATCCTCCTCCAGGACTC-3'
	Probe:	5'-CCAGGTGTGCGACTCTGACACT-3'
UDPGD	Left:	5'-AGATCCTCACCACTAATACTTGGTC-3'
	Right:	5'-AGAGCACTTATGGAGTTAATGCTG-3'
	Probe:	5'-GAGCTTTCCAAACTGGCAGCAAAT-3'

0.35±0.17% of total CD44 (*CD44-lt* + *CD44-st*). The percentage of exon 19 containing *CD44* mRNA increased up to 3.8% in the fourth and fifth weeks of culture (Fig. 1d). After seeding chondrocytes on 3D scaffolds, *CD44-lt* and *CD44-st* expression declined, especially in the first days. At day 16 of 3D culture, expression of *CD44-lt* and *CD44-st* was lower (3-fold and 5-fold, respectively) than in monolayer culture and showed almost no difference between the two scaffolds (Fig. 3).

FACS analysis of CD44 and CD90. An antibody recognizing all extracellular isoforms of CD44 was used to investigate CD44 expression by FACS. After two days and four weeks, monolayer culture chondrocytes showed similar CD44 expression (Fig. 4). To visualize the ongoing dedifferentiation of the chondrocytes, an antibody against CD90, a widely used fibroblast marker, was used. At day two, only minimal levels of CD90 expression were detectable, whereas, after four weeks, two cell populations, a slightly positive and a strongly positive, were apparent (Fig. 4).

Influence of cell density on CD44 mRNA expression. To determine a possible dependency of cell density on *CD44* expression, chondrocytes from four different patients were seeded on 24-well plates at four different cell densities ($5x10^3$, $2.5x10^4$, $1x10^5$ and $5x10^5$ cells per cm^2). Chondrocytes at

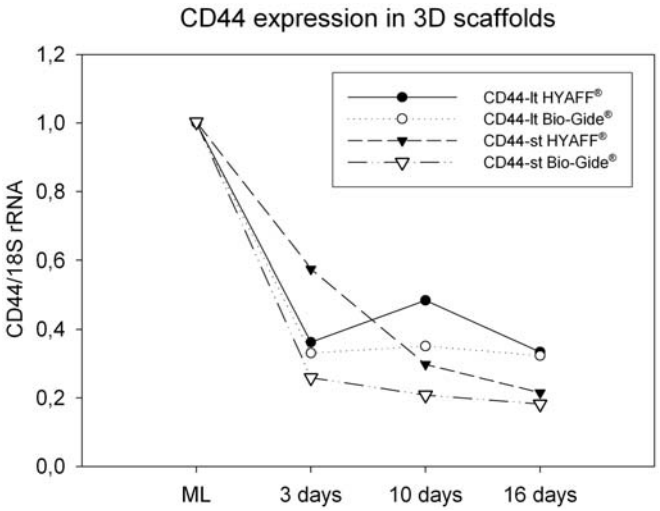

Figure 3. Real-time PCR analysis of *CD44-lt* and *CD44-st* in 3D scaffolds. For redifferentiation analysis dedifferentiated chondrocytes from monolayer culture (ML) were seeded on hyaluronan (HYAFF® 11) or porcine Collagen I/III (Bio-Gide®) scaffolds. Gene expression was normalized to *18S rRNA* expression. Data are expressed as mean ± SD of real-time PCR triplicate analysis.

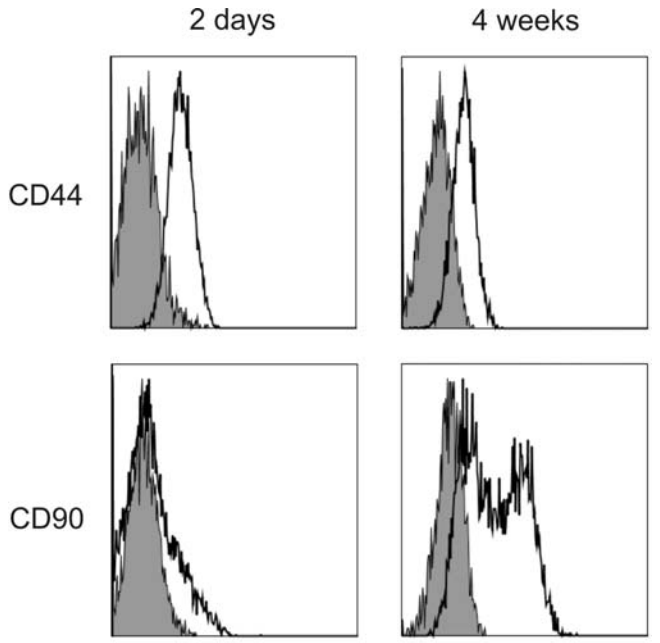

Figure 4. FACS analysis of CD44 and CD90. After two days and four weeks in monolayer culture, chondrocytes showed similar CD44 expression. CD90 expression was minimal at day two, whereas, after four weeks, one slightly positive and one strongly positive CD90 population were detectable. The results are presented as FACS histograms (isotype-control stain, grey histogram; surface marker stain, empty histogram).

$5x10^5$ cells per cm^2 showed significantly higher *CD44-lt* (2.1-fold, $p<0.03$) and *CD44-st* (2.2-fold, $p<0.03$) expression than cells at $5x10^3$ cells per cm^2 (Fig. 5).

mRNA expression of hyaluronan synthases and UDPGD with real-time PCR. In cultured chondrocytes, no major changes in *HAS2* expression were seen compared to native cartilage. Ligament fibroblasts, however, showed a 43-fold higher *HAS2*

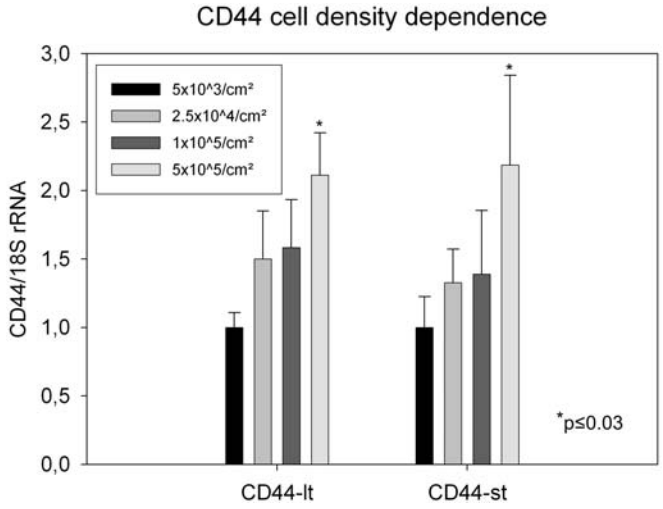

Figure 5. *CD44* cell density dependence. Chondrocytes were seeded at different cell densities in monolayer culture. Gene expression analysis showed a significant increase of *CD44-lt* ($p<0.03$) and *CD44-st* ($p<0.03$) expression at $5x10^5$ cells/cm^2 compared to $5x10^3$ cells/cm^2. Values were normalized to the expression of chondrocytes at $5x10^3$ cells/cm^2.

expression than native cartilage (Fig. 6a). *HAS3* expression varied greatly during cultivation time, but was usually within the range of native cartilage (Fig. 6b). In ligament fibroblasts, *HAS3* expression was approximately the same as in chondrocytes. The ratio of *HAS3* and *HAS2* ranged from 1.6 to 26.9% (Fig. 6c). Ligament fibroblasts were found at a rate of 1.8% in the lower level. *HAS1* was not detected in native cartilage nor cultivated chondrocytes or ligament fibroblasts.

Another important factor for hyaluronan synthesis, UDP-glucose dehydrogenase *(UDPGD)*, was found to be upregulated in the monolayer culture. After an increase of ~16-fold, compared to native cartilage in the first two days, *UDPGD* was expressed more or less constantly at a level 5-fold that of native cartilage (Fig. 6d).

Discussion

In order to obtain a sufficient amount of cells for matrix associated autologous chondrocyte transplantation (MACT), the isolated chondrocytes must be expanded in a monolayer culture. During this cultivation time, the chondrocytes undergo a dedifferentiation process, which enables the cells to proliferate. Previous studies that analyzed this dedifferentiation process primarily concentrated on matrix components, such as *collagen type I, II* and *aggrecan* expression (15,16). Nothing was known about alterations in *CD44*, particularly different *CD44* isoform expressions, or the genes that regulate the hyaluronan synthesis, although these genes play an essential role in matrix assembly and homeostasis.

A component of the extracellular matrix found in most vertebrate tissues is hyaluronan that is synthesized by a family of three different hyaluronan synthases (HAS1, HAS2, HAS3) at the cell surface (17-20). Depending on the HAS isoform, the synthesized hyaluronan polymers differ in size and molecular weight (21). While *HAS2* and *HAS3* have already been detected in human cartilage, the expression of *HAS1* is a subject of controversy (22,23). A further requirement for hyaluronan and other glycosaminoglycan synthesis is the availability of UDP-sugar. Prior to the

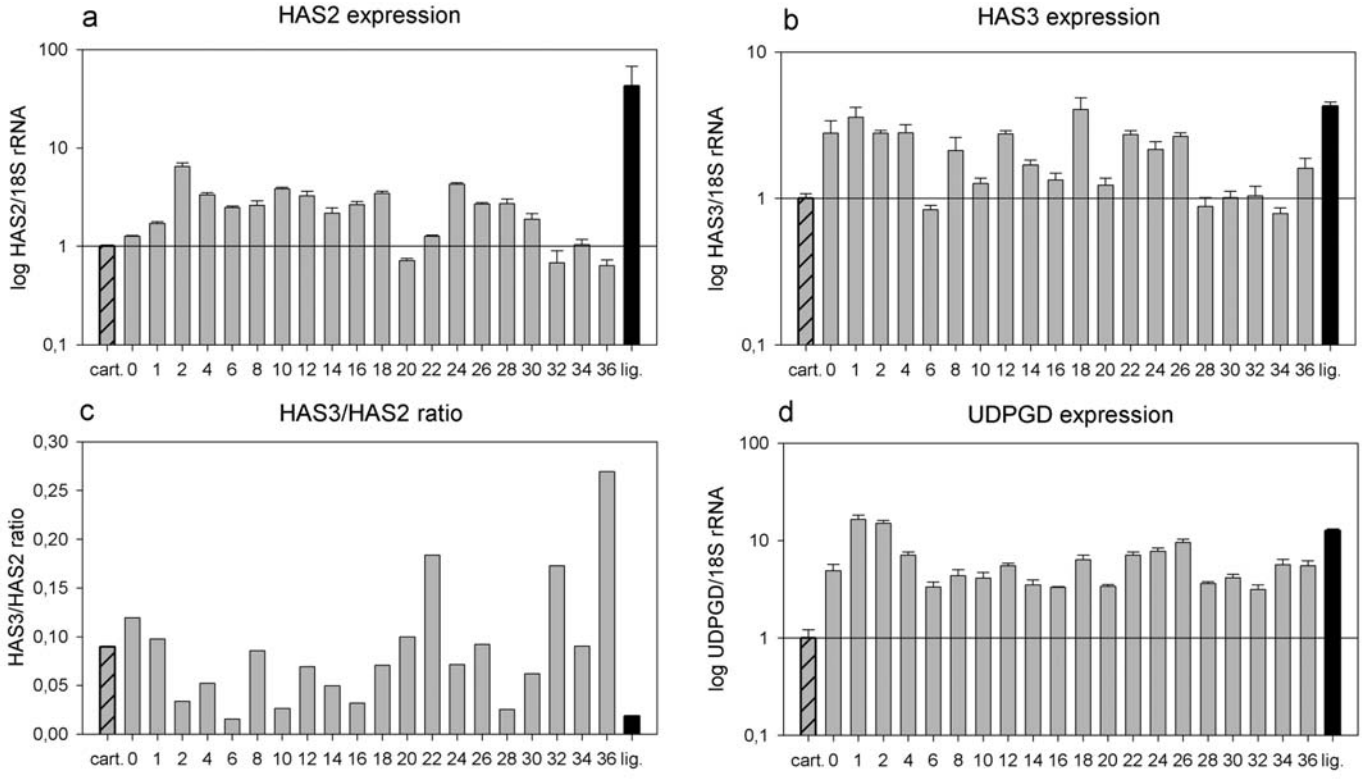

Figure 6. Real-time PCR analysis of *HAS* and *UDPGD* in native cartilage (cart.), chondrocytes in monolayer culture (day 0-36) and ligament fibroblasts (lig.). Gene expression was normalized to *18S rRNA* expression. Data were log transformed and expressed as mean ± SD of real-time PCR triplicate analysis.

synthesis of hyaluronan, UDP-glucose has to be converted to UDP-glucuronic acid by UDP-glucose dehydrogenase (UDPGD). This conversion seems to be a key regulator in the hyaluronan synthetic pathway (24-26).

In the present study, *HAS2* was found to be the predominant isoform in cartilage, dedifferentiated chondrocytes, and ligament fibroblasts, whereas *HAS3* was expressed at lower levels and *HAS1* could not be detected in any sample. When comparing chondrocytes in monolayer culture and native cartilage, *HAS* expression was very similar, only *UDPGD* was slightly upregulated in monolayer culture indicating that hyaluronan synthesis is only slightly affected by monolayer culture condition. The receptor of hyaluronan is CD44. In our study, *CD44s* was identified as the predominantly expressed *CD44* isoform in all samples.

In search of variant *CD44* exons RT-PCR revealed only one intense band at 400 bp, the expected size for *CD44s*, as well as a slight smear of higher molecular weight in all samples. Attempts to amplify other isoforms out of this smear by RT-PCR resulted always in the 400 bp product again. Taking into account that our real-time data showed a rather parallel course of *CD44s* and *CD44-lt*, we assume that *CD44-lt* corresponds to *CD44s*. We demonstrated that there was a distinct increase in *CD44s* expression immediately after the cell isolation. This high expression remained constantly during the entire cultivation time. We found that seeding density in monolayer culture positively correlates with *CD44* expression level. After seeding the chondrocytes into two different scaffolds that are commonly used for cartilage transplants (27,28) in order to induce redifferentiation and reduce proliferation, *CD44* expression was downregulated by day three. What could be the explanation of the increase of *CD44* in monolayer culture? A recent study by Tanimoto *et al* (29) demonstrated that blocking of CD44 led to a lower proliferation capacity of ligament fibroblasts *in vitro*. One could speculate that similar mechanisms are activated in chondrocytes. We do not assume that matrix production or assembly is dependent on the expression level of CD44 in 3D cultures. We rather speculate that there is a connection between the proliferation rate of mesenchymal cells and the expression level of CD44.

The expression of exon 19 rather than exon 20 gives rise to a CD44 isoform that lacks the cytoplasmic domain. We are not the first to document *CD44-st* expression. Goldstein *et al* detected short-tail isoform mRNA in several lymphoid as well as non-lymphoid cells, such as foreskin fibroblasts (30,31). In human chondrocytes, *CD44-st* was also discovered by Jiang *et al*, who reported a ratio of exon 19- to exon 20-containing *CD44* mRNA that varied from 15-33% (4). We observed a much lower level of *CD44-st* (0.19-0.4% of total CD44) in native cartilage, cultivated chondrocytes (up to three weeks in monolayer), and ligament fibroblasts. After three weeks in monolayer culture, CD44-st expression increased, whereas *CD44-lt* expression remained constant, resulting in a *CD44-st*/*CD44-lt* ratio of up to 3.8%. Although the *CD44-st*/*CD44-lt* ratio increased >10-fold, it was still much lower than that found by Jiang *et al* (4).

What are the factors that influence *CD44* expression, particularly *CD44-st* expression? It has been demonstrated that treatment of chondrocytes with interleukin-1α (IL-1α) resulted in an increase in *CD44-lt* and *CD44-st* expression, *CD44-st* being higher upregulated than *CD44-lt* (4). A multiplicity of functions were described for CD44, including the anchoring of hyaluronan to the cell surface, the mediation of hyaluronan endocytosis, cell proliferation, cell maturation, and cell migration (4,9,32). Nevertheless, the question is whether all these functions are mediated by the same CD44 isoform. Most studies did not discriminate between different CD44 isoforms, although these could give an explanation for the different results obtained. However, even studies that focused on CD44-st showed contradictory results. Although, in an overexpression model, CD44-st was demonstrated to inhibit the pericellular matrix (31), the antisense inhibition of CD44-st led to less cell-associated matrices and enhanced hyaluronan internalization (4). Thus, it is difficult to interpret the function and upregulation of *CD44-st* found in monolayer culture. At any rate, the existence of the *CD44-st* isoform, even in native cartilage, and the increase in monolayer culture, suggests a specific biological function for this isoform.

The fact that *CD44-st* expression is increased by IL-1α, a mediator of osteoarthritis, and by monolayer culture, indicates that *CD44-st* is expressed when ECM is degraded or not needed. Both osteoarthritis and chondrocytes in monolayer culture are associated with a higher cell proliferation rate than physiologically normal cartilage. Perhaps CD44-st has certain regulatory effect on CD44-lt that enables a switch in its function from matrix preservation toward proliferation.

We are rather optimistic that CD44 has a regulatory function in chondrocytes, not necessarily on differentiation, but possibly on proliferation. However, the nature of this influence remains to be determined.

Acknowledgements

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