In vitro expansion affects the response of chondrocytes to mechanical stimulation

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Summary

Objective: Expansion of autologous chondrocytes is a common step in procedures for cartilage defect repair. Subsequent dedifferentiation can alter cellular response to mechanical loading, having major consequences for the cell’s behavior in vivo after reimplantation. Therefore, we examined the response of primary and expanded human articular chondrocytes to mechanical loading.

Method: Primary and expanded chondrocytes were stretched at either 0.5% or 3.0% at 0.5 Hz, 2 h per day, for 3 days. Gene expression levels of matrix components (aggrecan (AGC1), lubricin (PRG4), collagen type I (COL1), type II (COL2) and type X (COL10)) as well as matrix enzymes (matrix metalloproteinase 1 (MMP1), MMP3, MMP13) and SOX9 were compared to unstretched controls. To evaluate the effect of a chondrogenic environment on cellular response to stretch, redifferentiation medium was used on expanded cells.

Results: In primary chondrocytes, stretch led to mild decreases in AGC1, COL1 and COL10 gene expression (maximum of 3.8-fold) and an up-regulation of PRG4 (2.0-fold). In expanded chondrocytes, expression was down-regulated for AGC1 (up to 21-fold), PRG4 (up to 5.0-fold), COL1 (10-fold), COL2 (2.9-fold). Also, expression was up-regulated for MMP1 (20-fold) and MMP3 (up to 4-fold), while MMP13 was down-regulated (2.8-fold). A chondrogenic environment appeared to temper effects of stretch.

Discussion: Our results show that expansion alters the response of human chondrocytes to stretch. Expanded chondrocytes greatly decrease gene expression of matrix constituents and increase expression of MMPs, whereas primary chondrocytes hardly respond. Our data could be a reference for optimization of cell sources or expansion protocols for reimplanted chondrocytes.

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Introduction

In autologous chondrocyte implantation (ACI) procedures, cartilage is harvested from an autologous donor site and isolated chondrocytes are expanded in vitro to obtain sufficient cell numbers before implantation into the defect site. However, during expansion culture, chondrocytes lose their specific chondrocytic phenotype and become more fibroblast-like. This phenotypical change, called dedifferentiation, is accompanied by a decreased gene expression of cartilage specific markers like collagen type II (COL2). This process might also alter the response of chondrocytes to extracellular stimuli. The current work studied the response of chondrocytes to mechanical stimulation after dedifferentiation resulting from monolayer expansion.

In their natural environment, chondrocytes are constantly deformed as a result of loading due to normal daily activities. Guilak et al. estimated the loss of cell height of chondrocytes resulting from physiological loading to be approximately 20%. In vivo deformation will also occur in reimplanted chondrocytes after ACI. Normal physiological loading is generally regarded as a prerequisite for the maintenance of proper articular joint functioning, while injurious loading can lead to cartilage degeneration. Dynamic compression of bovine explants or three-dimensional scaffold cultures has indeed shown a stimulatory effect in vitro, not only on load bearing matrix components, but recently also on lubricin. Other forms of mechanical stimulation like fluid flow induced shear stress and mechanical stretch also elicit a response in primary bovine chondrocytes. In human normal, healthy chondrocytes Millward-Sadler et al. found that cyclic stretch has an anabolic effect, as was shown by an increase in aggrecan (AGC1) expression and decrease in matrix metalloproteinase 3 (MMP3) expression. This effect was not seen in osteoarthritic (OA) chondrocytes, where no
change in AGC1 or MMP gene expression was observed. This difference might be attributed to a change in mechano-transduction pathways between normal and OA chondrocytes. In another study with human cartilage, Plumb and Aspden also showed that cyclic loading was not stimulatory in cartilage explants from human femoral heads. These results are contradictory to those found for young bovine chondrocytes, where loading was stimulatory.

Not only the source of chondrocytes determines the cell's response to mechanical loading. Wiseman et al. showed that bovine articular chondrocytes in agarose constructs exhibited decreased proliferation and proteoglycan synthesis after monolayer expansion upon mechanical stimulation compared to primary chondrocytes. Since expansion and the associated dedifferentiation of human chondrocytes is an essential step in ACI-like procedures, the effect of expansion on the matrix-forming capacities warrants further investigation.

Therefore, we investigated, through real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis, how human articular chondrocytes, after monolayer expansion, respond to stretch depending on their expansion and corresponding differentiation state. In addition, we examined whether a specific chondrogenic environment, which leads to redifferentiation to the chondrogenic phenotype, alters the response of expanded chondrocytes to stretch in terms of gene expression.

Methods

CELL CULTURE

Cartilage was obtained from patients undergoing total knee replacement surgery (after approval by the local ethical committee; MEC2004-322). Full thickness cartilage was harvested, treated with 0.2% protease in physiological saline solution (Sigma, St. Louis, MO, USA) for 90 min and subsequently digested overnight in basal medium [Dulbecco's modified eagle medium (DMEM), 4.5 g/l glucose with 10% Fetal Calf Serum (FCS), 0.1% gentamicine and 0.6% fungizone (both from Sigma, St. Louis, MO, USA), 0.1% gentamicine and 0.6% fungizone (both from Invitrogen, Scotland, UK)] supplemented with 0.15% insulin-like growth factor-1 (IGF-1), 25 μg/ml -ascorbic acid 2-phosphate (both from Sigma, St. Louis, MO, USA), 10 ng/ml transforming growth factor-β1 (TGF-β1) (recombinant human, R&D Systems, Abingdon, UK), 10 ng/ml insulin-like growth factor-1 (IGF-1), 25 μg/ml -ascorbic acid 2-phosphate (both from Sigma, St. Louis, MO, USA), 0.1% gentamicine and 0.6% fungizone (both from Invitrogen, Scotland, UK)].

MECHANICAL STIMULATION

Cells were left to adhere firmly to the flexible membrane of the six-well plate during a 5 day pre-culture with basal medium. On day 5, cells were stretched using a modified Flexcell set-up (Flexercell, McKeesport, PA, USA) inside an incubator (37°C, 5% CO2). This set-up was previously described. Briefly, a low pressure created under the six-well plates pulls the flexible membrane over a loading post, resulting in homogenous biaxial strain. The size of the loading post and the level of the pressure correlate to the amount of stretch applied to the adherent cells. Loading posts of 25 mm and 30 mm diameter were used, resulting in applied strains of 3.0% and 0.5%, respectively. Cyclic stretch at a frequency of 0.5 Hz was applied twice daily for 1 h with a 1 h rest period. This protocol was repeated for 3 days. Unstretched controls were placed in the device without stretching the membranes.

REDIFFERENTIATION MEDIUM

To examine the effects of a chondrogenic environment, experiments were also conducted with redifferentiation medium. This medium consisted of DMEM high glucose, 1:100 insulin-transferrin-selenium A supplement (ITS) – (BD Biosciences, Bedford, MA, USA), 10 ng/ml transforming growth factor-β1 (TGF-β1) (recombinant human, R&D Systems, Abingdon, UK), 10 ng/ml insulin-like growth factor-1 (IGF-1), 25 μg/ml -ascorbic acid 2-phosphate (both from Sigma, St. Louis, MO, USA), 0.1% gentamicine and 0.6% fungizone (both from Invitrogen, Scotland, UK). The redifferentiation medium was added at the onset of stretch.

PCR

Directly after the last stretch cycle total RNA was isolated using the Nucleospin II kit according to the manufacturer's instructions (Machery-Nagel, Düren, Germany) and nucleic acid content was determined spectrophotometrically (NanoDrop ND1000, Isogen Life Science, The Netherlands). For cDNA synthesis and real-time quantitative PCR (qPCR) methods see Uitterlinden et al. An ABI7000 was used for cycling. Taqman™ or SybrGreen™ assays were performed on AGC1, proteoglycan 4 (PRG4, alias lubricrin or superficial zone protein), COL1, COL2 and COL10, MMP1, MMP3, MMP13 and transcription factor (sex determining regionY) box 9 (SOX9), Glucosamine-6-sulfate deacetylase (GAPDH) was used for normalization. All primer and probe nucleotide sequences for gene amplifications are listed in Table I.

DATA ANALYSIS

Expression was normalized to GAPDH and expressed relatively using the 2^-ΔΔCt method of Livak. Subsequently, expression levels of unstretched control conditions were set to 1 and stretched conditions were plotted relative to controls. Results are means plus standard deviation. Statistical significance was determined using a Kruskal–Wallis test (SPSS Inc., Chicago, IL, USA) prior to testing stretched vs unstretched conditions by Mann–Whitney test. Differences were considered significant when P < 0.05. For every experiment with primary cells, six control wells were used for each donor, while three wells were used for 0.5% and three wells for 3.0% strain. The first experiment with expanded cells had the same set-up as the experiments with primary cells. For the other experiments with expanded cells, three wells were used for unstretched controls on basal medium and three wells were used for unstretched controls with redifferentiation medium. For both stretched conditions (0.5% and 3.0%), three wells per condition were used with basal medium and three wells were used with redifferentiation medium. Table II summarizes experimental details: some wells were lost due to low cell yield after harvest.

Results

EFFECT OF EXPANSION CULTURE ON THE LEVELS OF GENE EXPRESSION

Upon expansion in monolayer culture, gene expression of COL1 was up-regulated while SOX9 expression was down-regulated, typical for dedifferentiation toward a more fibroblast-like phenotype (Fig. 2). At the same time, COL2 is hardly expressed and COL10 expression is completely absent in dedifferentiated chondrocytes, also consistent with the shift toward a fibroblast-like state. Also, expression levels of MMP1, MMP3 and MMP13 were considerably lower after expansion.

EFFECT OF STRETCH ON PRIMARY CHONDROCYTES

Gene expression of matrix components (AGC1, PRG4, COL1, COL2 and COL10) was moderately altered by
stretch in P0 chondrocytes (Fig. 3). AGC1 and COL1 were down-regulated in a response to mechanical stimulation of both 0.5% and 3.0% strain levels. Gene expression of AGC1 was only slightly altered, a 1.8-fold down-regulation was found at 0.5% strain and 1.6-fold change at 3.0% strain. COL1 showed a 3.8-fold decrease in gene expression level compared to control at 0.5% strain and a 2.1-fold decrease at 3.0% strain. COL10 was also marginally down-regulated (2-fold) at both strain levels. Gene expression levels of COL2 remained unaltered when loaded with either 0.5% or 3.0% strain, while levels of PRG4 were slightly up-regulated compared to control, 1.8-fold at 0.5% and 1.9-fold at 3.0%.

Stretch did neither statistically significantly alter the gene expression of MMP1, MMP3 and MMP13, nor did it change SOX9 expression levels.

EFFECT OF STRETCH ON EXPANDED CHONDROCYTES

P3 cells showed a much larger response to stretch (Fig. 4) than primary cells. Gene expression of most matrix proteins (AGC1, PRG4, COL1 and COL2) were severely down-regulated after stretching of the cells. AGC1 and COL1 showed the most significant change in gene expression. Expression levels of AGC1 were 15.6-fold lower at 0.5% when compared to unstretched controls, while 3.0% resulted in a 11-fold decrease. COL1 was down-regulated approximately 10-fold for both 0.5% (11.9-fold) and 3.0% strains (8.0-fold). Gene expression of PRG4 was also lower when cells were stretched at 0.5% (5.0-fold decrease) or 3.0% (2.5-fold decrease). After expansion, COL2 expression was absent in chondrocytes from one donor. In those cases where COL2 was still expressed, stretching down-regulated its expression levels (up to 2.9-fold for 0.5% stretch). COL10 was not expressed in any donor after expansion.

In expanded chondrocytes, MMP1 and MMP3 were both up-regulated after stretching. MMP1 showed a 20-fold up-regulation while MMP3 was up-regulated 3.5-fold (at 0.5% strain) or 2.3-fold (at 3.0% strain). MMP13 was down-regulated in response to cell straining at both 0.5% (2.8-fold) and 3.0% (1.9-fold). Again, no effect of stretch on SOX9 gene was found.

EFFECT OF STRETCH ON EXPANDED CELLS IN A CHONDROGENIC ENVIRONMENT

On redifferentiation medium, expanded chondrocytes re-expressed COL2 and COL10, indicating a return to a more...
chondrogenic phenotype. COL1 and PRG4 expression was also higher on redifferentiation medium. Gene expression of MMP1 and MMP13 was also up-regulated on redifferentiation medium.

This chondrogenic environment did not significantly change the alterations in gene expression levels of matrix components by expanded chondrocytes associated with stretch (Fig. 5). AGC1 and COL1 were still severely down-regulated, while PRG4 was again only moderately down-regulated. COL2 was re-expressed on redifferentiation medium, but here stretch also appeared to down-regulate gene expression. No effect of stretch was found on mRNA levels of COL10 gene expression. MMP1 expression was still up-regulated, but to a lesser extent compared to basal medium. MMP3 expression was still significantly up-regulated in stretched conditions compared to unstretched controls on redifferentiation medium. MMP13 was no longer significantly down-regulated. Overall an expression pattern was found that was similar to that found with basal expansion medium, but the effects seemed somewhat tempered.

General trends for all conditions are summarized in Table III.

Discussion

Our results indicate that in vitro expansion affects the response of chondrocytes to a mechanical stretch protocol. Real-time RT-PCR analysis revealed a decrease in expression of genes encoding for matrix components as well as a rise in expression of matrix degrading enzymes after stretching of expanded chondrocytes. In primary chondrocytes the response was markedly less substantial and significant. We also studied the effect of a chondrogenic environment that is known to direct dedifferentiated chondrocytes back toward a chondrogenic phenotype. These partially redifferentiated chondrocytes showed similar effects as the expanded, dedifferentiated chondrocytes, however, the effects of stretch appeared to be tempered. This is consistent with the shift toward the primary phenotype, since primary chondrocytes reacted only marginally to stretch.

The observation of up-regulation of the matrix degrading enzymes MMP1 and MMP3 after stretch is consistent with the notion that chondrocytes assume a more fibroblast-like
phenotype upon dedifferentiation in respect that some studies report increased (pro-)MMP expression following (injurious) loading. For example, increased (pro-)MMP expression and activation was found in ligament fibroblasts, patellar tendon fibroblasts, scleral fibroblasts, uterine cervical fibroblasts and cardiac fibroblasts after loading with stretch. However, this up-regulation was not found in all types of fibroblast. Sambajon et al.31 found no difference in proteinase activity of synovial fibroblasts after stretch. However, matrix degradation is also part of the remodeling process and it cannot be excluded that the rise in expression of MMP1 and MMP3 after short-term stretch follows from a remodeling attempt by the cells. But one might expect a concurrent elevation in matrix components in case of remodeling, which is not seen in our experiments.

Interestingly, expression levels of MMP13, the collagenase whose affinity for COL2 is the greatest, were down-regulated after cyclic stretch. This difference in response might be attributed to the fact that the collagenases MMP1 and MMP13 differ in their spatial distribution. MMP1 is mainly expressed in the superficial cartilage layer, while MMP13 is chiefly expressed in the deep zone, where different deformation is experienced by the chondrocytes.

Other than very marginal changes in the expression of AGC1 and COL1 and COL10, primary chondrocytes did not show a marked response to stretch. This is in line with the findings of Millward-Sadler et al.17, who found that expression levels of AGC1, MMP1 and MMP3 were unchanged in primary chondrocytes from OA patients after application of short-term cyclic stretch. The discrepancy with primary chondrocytes from healthy cartilage, which showed an increase in AGC1 expression and a decrease in MMP3 expression, was attributed to phenotypical alterations in OA chondrocytes. In OA chondrocytes, these changes might include altered expression of integrins, cytokines and growth factors. Indeed, integrins, and especially the fibronectin receptor integrin α5β1, are involved in mechanotransduction of both normal and OA chondrocytes. Although the exact mechanisms by which this transduction occurs are not yet fully understood, they appear to include initiation of integrin-dependent signaling cascades. Expression of...
The expression response after stretch, although the effects of this altered gene might not be the optimal choice for such a procedure. Consequently, it might be that other cell sources, redifferentiation protocols prior to implantation or limiting deformation (e.g., by movement restricting post-surgical therapy or use of a rigid scaffold) improve the cell’s capacity to form a functional extracellular matrix and reduce enzymatic activity. However, implanted chondrocytes should also become involved in remodeling of the matrix, starting with degradation that might lead to better incorporation with the host matrix. From the current study, providing short-term RNA-level responses, we have no information regarding the long-term consequences for the matrix and its in vivo incorporation potential. Our findings may therefore be regarded as a reference point for future studies that aim to optimize protocols for tissue formation by expanded chondrocytes.

### References


