Osteoarthritis and Cartilage (2008) **16,** 385–391

doi:10.1016/j.joca.2007.07.014

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

In vitro expansion affects the response of chondrocytes to mechanical stimulation[1]

R. H. J. Das M.Sc.†, H. Jahr Ph.D.†, J. A. N. Verhaar M.D., Ph.D.†, J. C. van der Linden Ph.D.†‡, G. J. V. M. van Osch Ph.D.†§ and H. Weinans Ph.D.†*

† *Department of Orthopaedics, Erasmus MC, University Medical Center Rotterdam, P.O. Box 2040, 3000 CA, The Netherlands*

‡ *Faculty of Mechanical Engineering, Department of Precision and Microsystems Engineering, Delft University of Technology, Delft, Mekelweg 2, 2628 CD, The Netherlands*

§ *Department of Otorhinolaryngology, Erasmus MC, University Medical Center Rotterdam, P.O. Box 2040, 3000 CA, The Netherlands*

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) and 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.

Key words: Cell culture, Mechanical loading, Chondrocyte phenotype, Dedifferentiation, Gene expression, Cell deformation, Stretch, Tissue engineering.

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[1,2]. This phenotypical change, called dedifferentiation, is accompanied by a decreased gene expression of cartilage specific markers like collagen type II (COL2)[3]. 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.*[4] 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[5–11], but recently also on lubricin (PRG4)[12]. Other forms of mechanical stimulation like fluid flow induced shear stress[13,14] and mechanical stretch[15,16] also elicit a response in primary bovine chondrocytes. In human normal, healthy chondrocytes Millward-Sadler *et al.*[17] 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

[1]This research was funded by the Dutch Program Tissue Engineering (DPTE, Project Number RGT6738).

*Address correspondence and reprint requests to: Dr Harrie Weinans, Ph.D., Orthopaedic Research Laboratory, Erasmus MC, Room EE-1614, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands, Tel: 31-10-4087367; Fax: 31-10-4089415; E-mail: h.weinans@erasmusmc.nl

Received 11 January 2007; revision accepted 29 July 2007.

change in AGC1 or MMP gene expression was observed. This difference might be attributed to a change in mechanotransduction pathways between normal and OA chondrocytes[18–20]. In another study with human cartilage, Plumb and Aspden[21] 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[5–7].

Not only the source of chondrocytes determines the cell's response to mechanical loading. Wiseman *et al.*[22] 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 (all Invitrogen, Scotland, UK)] supplemented with 0.15% collagenase B (Roche Diagnostics, Mannheim, Germany). The following day, the harvested cell number was determined using a heamacytometer. The primary chondrocytes were then either seeded at a density of 7500 cells/cm^2 in a T175 culture flask for expansion culture or seeded at a density of 300,000 cells/well in collagen type I (COL1) coated Flexcell six-well plates (Flexercell, McKeesport, PA, USA). The cells plated for expansion were cultured for three passages. These expanded chondrocytes were then seeded at a density of 300,000 cells/well in the Flexcell COL1 coated six-well plates (Fig. 1).

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% CO_2). This set-up was previously described[23]. 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.

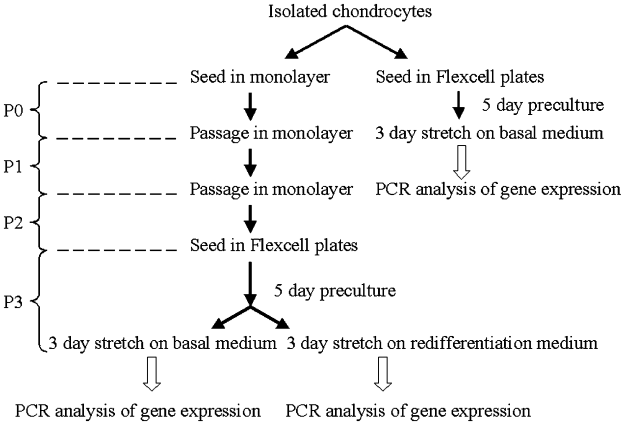

Fig. 1. Layout of experimental set-up.

REDIFFERENTIATION MEDIUM

To examine the effects of a chondrogenic environment, experiments were also conducted with redifferentiation medium[2]. 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-β2 (TGF-β2) (recombinant human, R&D Systems, Abington, UK), 10 ng/ml insulin-like growth factor-1 (IGF-1), 25 μg/ml L-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.*[24]. An ABI7000 was used for cycling.

Taqman™ or SybrGreen™ I assays were performed on AGC1, proteoglycan 4 (PRG4, alias lubricin or superficial zone protein), COL1, COL2 and COL10, MMP1, MMP3, MMP13 and transcription factor (sex determining regionY)-box 9 (SOX9). Glyceraldehyde-3-phosphate dehydrogenase (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 $2^{-(\Delta\Delta Ct)}$ method of Livak[25]. 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 plate 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