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Altered *in vitro* chondrogenic properties of chondrocytes harvested from unaffected cartilage in osteoarthritic joints[1]

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Summary

Objective: *In vitro* models of chondrogenesis often depart from chondrocytes harvested from less-affected areas of osteoarthritic joints. However, there are indications that these chondrocytes are phenotypically different from chondrocytes from healthy joints and thus might differ in their capacity to generate hyaline cartilage. The goal of this study was to compare the chondrogenic capacity of chondrocytes from healthy and OA joints.

Design: Chondrocytes isolated from nine healthy and nine OA knee joints were expanded in monolayer for two passages. Chondrocytes from passages 1 and 2 were analyzed for expression of (de)differentiation and hypertrophy markers and were seeded at passage 2 on collagen-coated filters for redifferentiation culture to study cartilage matrix formation.

Results: The collagen II/I mRNA ratio, reflecting differentiation, decreased from passage 1 to 2 in both chondrocytes from OA joints and chondrocytes from healthy joints ($P < 0.05$), without a significant difference between the two donor types. At passage 1, levels of the cartilage transcription factors Sox-5, Sox-6 and Sox-9 appeared to be higher in chondrocytes from OA joints (n.s.), but this was not seen at passage 2. However, a clear difference was observed in collagen type X expression, which was high in chondrocytes from OA joints at both passages, while undetectable in chondrocytes from healthy joints ($P < 0.01$). Tissue generated by chondrocytes from healthy joints redifferentiated for 28 days, showed a significantly better morphology, as assessed by histological scoring ($P < 0.01$) and higher proteoglycan content ($P < 0.05$), compared to chondrocytes from OA joints. Matrix turnover parameters, i.e., proteoglycan synthesis and degradation rate, were not significantly affected by donor tissue origin.

Conclusions: These results suggest that clear differences between chondrocytes from healthy and OA joints exist and that these are not completely abolished during the process of de- and redifferentiation. Therefore, *in vitro* cartilage regeneration models, which use chondrocytes from OA joints, should be interpreted with care.

Key words: Cartilage, Tissue engineering, Osteoarthritis, Chondrocytes, Culture technique.

Introduction

Autologous chondrocyte transplantation (ACT) has been shown to be a promising technique for the treatment of cartilage defects. However, the quality of the tissue synthesized *in vivo* still varies greatly, ranging from hyaline cartilage to fibrocartilaginous and hypertrophic tissue[1–3]. Hence, a great deal of research is being carried out to study and optimize cartilage regeneration, both *in vivo* and *in vitro*.

As in ACT, *in vitro* cartilage regeneration models depart from chondrocytes isolated from cartilage biopsies and expanded in monolayer. During this process, chondrocytes dedifferentiate and start producing aspecific extracellular matrix proteins, such as collagen type I[4,5]. *In vitro* redifferentiation is subsequently achieved, usually by seeding the dedifferentiated cells at high density in medium with appropriate factors. Frequently, chondrocytes used for *in vitro* research are harvested from less-affected areas of osteoarthritic joints, as this is a readily available source of cells. However, it is not clear whether these chondrocytes are actually phenotypically identical to chondrocytes from healthy joints. Transformation to osteoarthritic tissue is not very likely to involve sudden changes, but is more likely to be a gradual and initially microscopically invisible process. Although it may be argued that dedifferentiation, occurring as a result of expansion of harvested chondrocytes, effaces existing differences, leading to identical tissue compositions upon regeneration, it has never been investigated whether this is actually the case. Chondrocytes harvested from "healthy" areas of osteoarthritic joints may already display characteristics of chondrocytes in osteoarthritic lesions. These characteristics are an increased proteoglycan turnover rate, resulting from enhanced proteoglycan synthesis

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and release[6–11], the production of the hypertrophic marker type X collagen, and the expression of matrix degrading proteases such as cathepsin B and matrix metalloproteinase (MMP)-13. Moreover, chondrocytes from OA joints display a shift towards the production of collagen type I at the expense of collagen type II. As the chondrocyte phenotype, in particular the expression of collagen II and aggrecan, is dependent on the expression of the transcription factors L-Sox-5, Sox-6 and Sox-9, the diminished production of cartilaginous matrix proteins by the OA chondrocyte might be associated with decreased levels of these transcription factors.

The aim of the current study was to investigate to what extent chondrocytes harvested from OA joints display an altered, more dedifferentiated or hypertrophic, phenotype after expansion, compared to chondrocytes from healthy joints and to what extent this is associated with differences in cartilage regeneration upon dedifferentiation in monolayer and redifferentiation in a previously described *in vitro* model[12].

Materials and methods

CHONDROCYTE ISOLATION AND EXPANSION

Articular cartilage was harvested from healthy and osteoarthritic joints. From healthy knee joints cartilage was harvested postmortem (within 24 h after death) from femoral condyles of nine human donors, average age 50.3 years (38–68), five males and four females. From osteoarthritic knee joints, during joint replacement surgery, cartilage was harvested from femoral condyles of nine human donors, average age 65 years (57–73), three males and six females. Only cartilage that macroscopically looked relatively normal was used for this study, but cartilage biopsies from OA donors were more yellowish and had a softer texture compared to cartilage from healthy knee joints. No fibrous scar-like tissue was used. As all tissue was needed for adequate culture volumes, no tissue could be stored for histology. Therefore the morphological quality of the tissue could not be determined. In a separate study, tissue was harvested in an identical manner and an average modified Mankin score of 5.3 ± 2.8 points was observed ($0 =$ normal, $11 =$ complete degeneration[13]) (unpublished results). Exclusion criteria were a clinical history of inflammatory or crystalline joint disorders and usage of corticosteroids (systemically or intra-articularly) within 1 month before biopsy. The biopsies were transported in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisly, UK) containing 4.5 mg/ml glucose, L-glutamine, 10% fetal bovine serum (FBS) (Gibco), 1× nonessential amino acids (Sigma, St Louis, USA), 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) buffer (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 0.2 mM L-ascorbic acid (Sigma) and 0.4 mM proline (Sigma) at 4 °C.

Within 12 h after biopsy, chondrocytes were isolated by a 3-h 0.1% pronase (Roche, Mannheim, Germany) digestion followed by an over-night 0.04% collagenase (Sigma) digestion at 37 °C. Undigested debris was removed using a 100 μm cell strainer (Becton Dickson, Franklin Lakes, USA). The cells were washed once in phosphate buffered saline (PBS) and brought into suspension in expansion medium consisting of DMEM (Gibco) containing 4.5 mg/ml glucose, 10 mM HEPES buffer, 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Subsequently, the chondrocytes were seeded in monolayer at a cell density of 5000 cells/cm^2 and cultured in expansion medium. The expansion medium was supplemented with 10 ng/ml basic fibroblast growth factor (bFGF) (R&D, Minneapolis, MN, USA), except for the first 3 days of culture. The cells were cultured at 37 °C and 5% CO_2 for two passages. The culture medium was renewed every 3 days. At confluency the cells were trypsinized using 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) (Gibco) and were replated. The viable cells were counted using a Bürker Türk hemacytometer after staining with Trypan Blue. These cell numbers were used to calculate the number of population doublings (PDs) that occurred during expansion and the amount of cell suspension required for further expansion and/or redifferentiation. Part of the cells was harvested for mRNA extraction and ensuing polymerase chain reaction (PCR), while the remainder was redifferentiated for 28 days to determine the chondrogenic capacity.

REDIFFERENTIATION CULTURE

After the two passages, the cells were cultured in a previously published redifferentiation model in which the chondrocytes are seeded at a density of 1.6×10^6 cells/cm^2 (720,000 cells/filter) on millicell filters (Millipore Co, Bedford, MA, USA) that were precoated with collagen type II (Sigma)[12]. The original model has been adapted by eliminating serum, *N*-(2-hydroxyethyl)piperazine-*N*′-[2-ethanesulfonic acid] and β-glycerolphosphate, in order not to direct the redifferentiating chondrocytes towards a hypertrophic pathway. Instead, the cells were cultured in a redifferentiation medium consisting of DMEM (Gibco) supplemented with 10% human serum albumin (Equitech-Bio, Kerrville, TX, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.4 mM ascorbic acid, 1× ITSx (Gibco) and 5 ng/ml TGF-β2 (R&D). The cultures were incubated at 37 °C in 5% CO_2. The culture media were renewed every 3 days. This culture model with above described adaptations has been validated in a separate study (unpublished results).

HISTOLOGY

After 28 days of culture, samples were fixed in 10% buffered formalin for histological evaluation (Safranin O/Fast green). Sections were cut at 5 μm and stained with Safranin O/Fast Green. The histological quality of the tissue was analyzed by four blinded observers using a scoring system specifically designed to evaluate cartilage synthesized *in vitro* (obtained through personal communication). This scoring system will be further referred to as the "Bern Score"[14]. This scoring system evaluates uniformity and intensity of Safranin O staining, the distance between cells, the amount of matrix produced and cell morphology. Each item can be given a maximum of 3 points, thus optimal tissue quality scores 9 points.

IMMUNOHISTOCHEMISTRY

After 28 days of culture, samples were embedded in TissueTek O.C.T. compound (Sakura, Torrance, CA, USA), cut at 5 μm, and stained immunohistochemically for collagen type I and type II to assess the degree of redifferentiation. For immunohistochemical staining, the sections were incubated with monoclonal antibodies against collagen type I and type II (1:1500 Omnilabo and 1:100 Developmental Studies Hybridoma Bank, respectively) for 1 h at room temperature (RT). Subsequently the samples were incubated with a biotinylated goat anti-mouse secondary