Investigation of Collagen Transplants Seeded with Human Autologous Chondrocytes at the Time of Transplantation

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Abstract

Objective: The treatment of cartilage defects with matrix-embedded autologous chondrocytes is a promising method to support the repair process. In this study we gathered quality parameters of collagen I matrices and embedded autologous chondrocytes at the time of transplantation We determined number, morphology, and distribution of matrix-embedded chondrocytes as well as their synthesis performance concerning sulphated glycosaminoglycans (sGAG) and collagen 1A1 and 2A1 mRNA levels. *Results:* Chondrocytes were equidistantly distributed in the collagen matrices, and cell numbers ranged from 6 to 34×10^4 cells/g wet weight. Significant amounts of sGAG were detected in all of the investigated transplants but did not correlate with the number of cells within the respective transplants. Moreover, collagen I mRNA levels exceeded that of collagen II up to 17-fold. Collagen I and II ratio and sGAG amounts indicated significant interindividual differences of chondrocytes. The variation of transplant-associated sGAG levels could be attributed to the differential biosynthesis performance of chondrocytes. *Conclusions:* These results confirm the vitality and the chondrocytic phenotype of matrix-embedded cells (CaRes®) with respect to sGAG synthesis. However, chondrocytes showed collagen I mRNA expression partially far exceeding that of collagen II, indicating a rather dedifferentiated cellular status. In addition, sGAG synthesis performance of different patients' chondrocytes varied significantly. Nevertheless, a 2-year clinical study of chondrocyte-seeded collagen matrices as investigated in this work delivered promising results. However, future studies are planned to determine markers for the regenerative potential of donor chondrocytes.

Keywords

chondrocytes, articular cartilage, biomaterials, repair, tissue engineering

Introduction

The inherently limited capacity of damaged articular cartilage to heal or regenerate renders it an important objective for tissue engineering approaches. The latter include the employment of scaffolds seeded with autologous chondrocytes for replacement of damaged tissue parts. For development and implementation of the appropriate scaffold, the anatomical and physiological peculiarities of articular cartilage and its feasibility in clinical practice have to be considered. In terms of chemical composition and fabrication design, various materials meet the requirements by providing biophysical and biochemical properties similar to those of the cartilaginous extracellular matrix (ECM) and have already been applied in the field of orthopedic surgery (reviewed by Raghunath *et al.*[1]). Among the natural ones, collagen scaffolds have been prominent in the past 2 decades.[2] Besides proteoglycans, collagen II is one of the main constituents of articular cartilage comprising a crucial component of the microenvironment of chondrocytes. Collagen scaffolds hence afford chondrocyte-ECM interactions similar to those occurring in native cartilage and thereby contribute to the maintenance of the chondrocytic phenotype of cells.[3,4] Moreover, degradability of the collagen scaffold and the integration of embedded chondrocytes into the signaling network of the synovial cavity might provide the basis for the constitution of cartilage-equivalent tissue.

Although cell-seeded matrices are routinely used for treatment of cartilage defects, the embedded cells at the point in time of transplantation have not been investigated yet.

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In this study, we focused on the determination of some key parameters accounting for the quality of collagen I matrices seeded with autologous chondrocytes and their variability between transplants of different patients. For that, we determined the number and spatial distribution of transplant-embedded chondrocytes as well as their viability after isolation. To evaluate the chondrocytic phenotype, we determined the amount of sulphated glycosaminoglycans (sGAG) in transplants of different patients. Furthermore, we monitored the proliferation of isolated chondrocytes as well as their sGAG synthesis performance in the course of an 8-day cultivation period. This approach proved to facilitate the detection of interindividual differences with respect to proliferation and the discrimination between the sGAG fraction retained at the cell surface and that released into the medium. The ratio of the different sGAG fractions and the amount of cell-associated sGAG might additionally provide indications for the presence of chondrocyte-specific matrix-constituting binding partners of proteoglycans such as hyaluronan and/or collagens. In addition, the expression of collagen I and collagen II by matrix-embedded cells was determined at the mRNA level.

Materials and Methods

Autologous Chondrocytes-Seeded Collagen I Matrices

Autologous chondrocytes-seeded collagen matrices (CaRes®) were provided by Arthro Kinetics (Esslingen, Germany). Fabrication in brief: Chondrocytes for matrix embedment were derived from biopsies of patients with cartilage defects. Cells were isolated, seeded into a collagen matrix, cultivated in medium containing autologous serum for 10 to 14 days, and subsequently delivered as ready-to-use transplants to the clinician. A second transplant destined for reserve is routinely fabricated for each patient. Unused transplants were used for investigations.

Chemicals and Reagents

All chemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise. DMEM/F12 was from Invitrogen (Carlsbad, CA), and FCS was from PAA (Pasching, Austria). Kits for RNA extraction from paraffin-embedded slices, cDNA synthesis, and reverse transcriptase polymerase chain reaction (RT-PCR) were from Roche (Mannheim, Germany).

Isolation and Cultivation of Chondrocytes from Collagen Transplants

Autologous transplants were minced and incubated in DMEM/F12 containing collagenase II (100 U/mL medium)

until being completely digested (~3 hours). The cell suspension was passed through a 40-μm filter (BD, Franklin Lakes, NJ) to remove undigested debris, washed with phosphate-buffered saline (PBS), centrifuged (5 minutes, 1,000 rpm, room temperature [rt]), and resuspended in PBS. Viability was determined via trypan blue staining, and cells were counted using a hemocytometer prior to seeding chondrocytes into 6-well plates (Nunc, Rochester, NY) in a density of about 10^5 cells/cm^2.

Measurement of sGAG

Quantification of sGAG was conducted according to Barbosa *et al.*[5] In brief, cell culture supernatant was treated with proteinase K (50 μg/mL) at 56 °C overnight. After inactivation of the enzyme (90 °C, 10 minutes) and centrifugation (12,300 rpm, 4 minutes, RT), the supernatant was filtered through an ultrafree filter of 0.1-μm pore size (Millipore, Billerica, MA; 12,300 rpm, 4 minutes). One milliliter of a 1,9-dimethyl-methylene blue solution (DMMB) was added to 100 μL filtrate and vigorously mixed to allow the formation of complexes of DMMB and sGAG in the sample. The complexes were pelleted via centrifugation (12,300 rpm, 10 minutes, RT) and subsequently dissolved in decomplexation solution. After 30 minutes of shaking, the absorbance at 656 nm was measured photometrically. The sGAG amount was calculated from a standard curve with shark chondroitin sulphate. For determination of the amount of cell-associated sGAG, cells were scraped from the surface of the cell culture device and centrifuged (1,000 rpm, 5 minutes, RT). The cell pellet was resuspended in PBS, digested, and processed as described before. All experiments were done in triplicate.

Histology

For visualization of chondrocytes, the collagen matrix was fixed in 10% buffered formalin (VWR) for 24 to 48 hours at RT, dehydrated by serial incubations in alcohol and xylene, embedded in paraffin, sectioned at 5 μm, and processed for Azan staining. Images were taken with an Olympus IMT-2 microscope and processed using the Analysis SIS Five software.

RNA Isolation from Histologic Slices and RT-PCR

Isolation of total RNA from histologic slices was performed with the High Pure RNA Paraffin Kit according to the manufacturer's instructions. In brief, 3 formalin-fixed, paraffin-embedded slices of 10-μm thickness of the respective transplant were collected in reaction tubes and deparaffinized. The specimens were digested with proteinase K and the lysates transferred to filter tubes for extraction of nucleic acids. After digestion with DNase I, total RNA was

eluted from the filter membrane and collected. Reverse transcription was performed with the 1st Strand cDNA Synthesis Kit for RT-PCR, and PCR was done using FastStart TaqMan® Probe Master for quantification of Collagen I and Collagen II transcripts. Experiments were done in triplicate.

Results

Spatial Distribution and Morphology of Chondrocytes in Transplants

To determine morphology and spatial arrangement of chondrocytes, specimens of 8 different transplants were processed for Azan staining of histological slices. Thereby, cell nuclei appear in red with less pronounced staining of the cytoplasm and collagen in blue. All of the investigated transplants showed a similar distribution and morphology of embedded chondrocytes, as exemplified in **Figure 1**. They were more or less equidistantly distributed in the loose collagen-fibrous meshwork and appeared spindle shaped rather than spheroidal or oval as is typical for chondrocytes in native cartilage. Interestingly, we found only single cells but no cell clusters. Proliferation took place as determined as part of the transplant manufacturer's quality control. The numbers of cells isolated from the respective ready-to-use transplants ranged from 6 to 34 $\times$ 10^4 cells/g wet weight (**Fig. 2**), and cell viability was near 100% as determined by trypan blue staining (data not shown).

Expression of the Chondrocytic Phenotype by Matrix-Embedded Cells

As parameters of the chondrocytic phenotype of matrix-embedded chondrocytes, we determined the amount of sGAG as well as the ratio of collagen I and collagen II mRNA. The sGAG content associated with the different transplants ranged from 0.8 to 2.9 µg/g wet weight (**Fig. 3**) but did not correlate with the number of cells, indicating differences with respect to their biosynthetic performance. The amount of mRNA of collagen I and II was determined via RT-PCR using total RNA directly isolated from paraffin-embedded histologic slices of transplants. The ratio of mRNA expression levels of collagen I and collagen II (**Fig. 4**) of the different transplants ranged from 3 to 17.

Isolated Chondrocytes: Proliferation, sGAG Synthesis (Cell Associated versus Supernatant)

Chondrocytes isolated from 5 transplants (**Fig. 5A-E**) were further cultivated for 8 days under standard cell culture conditions. **Figure 5** depicts the numbers of originally seeded cells and the cell numbers after an 8-day cultivation
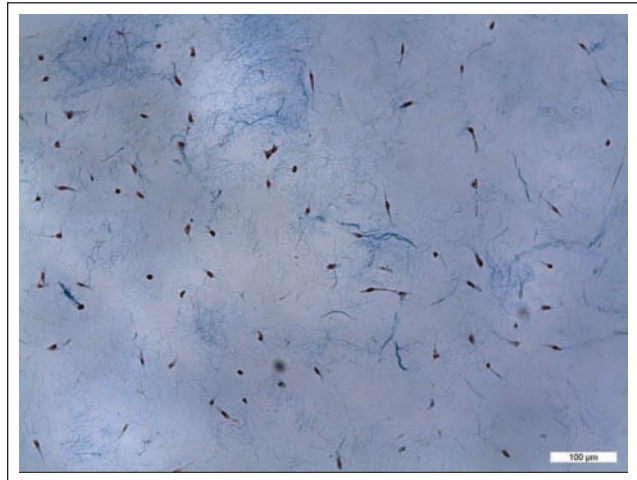

Figure 1. Distribution and morphology of chondrocytes in a collagen matrix. The matrix was histologically processed for Azan staining. Collagen fibers appear in blue, cells in red. Scale bar = 100 µm.
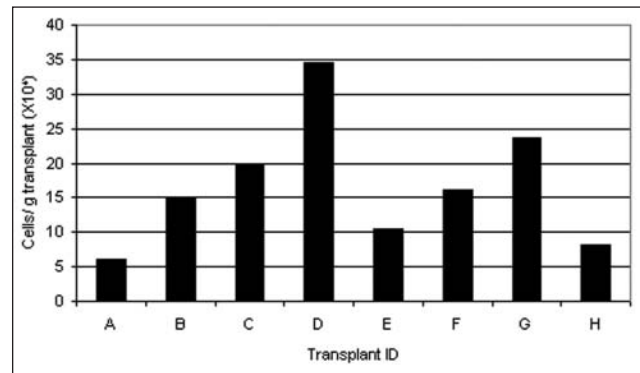

Figure 2. Number of chondrocytes isolated from 8 transplants (**A-H**).
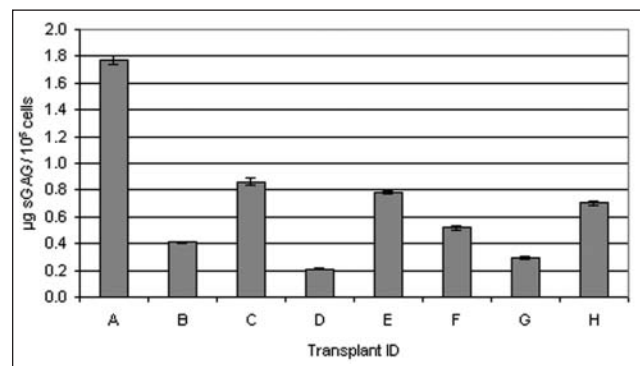

Figure 3. Amount of sGAG related to cell numbers of 8 transplants (**A-H**).

period. Pronounced differences in the proliferation rate of chondrocytes from different patients were obvious. Furthermore, we monitored sGAG synthesis performance and the capacity of cells to retain newly synthesized
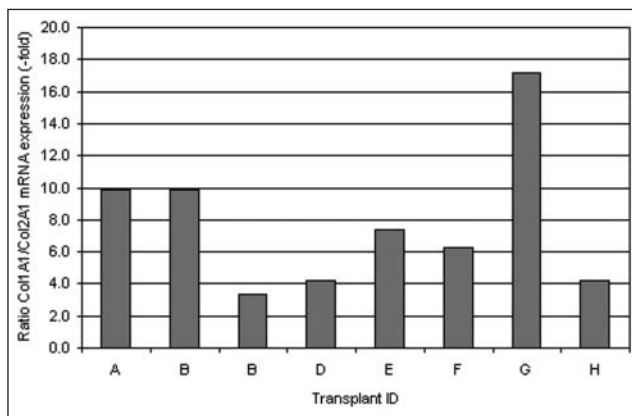
Figure 4. Ratio of Col1A1 and Col2A1 mRNA amounts of chondrocytes of 8 transplants (**A-H**).

Figure 5. Proliferation on cell culture conditions after isolation of cells from 5 different transplants (**A-E**). Number of originally seeded cells (gray bars) and after a 7-day cultivation period (black bars).

proteoglycans (sGAG) at the cell surface. In general, just a small proportion of the overall synthesized sGAG remained cell associated, and absolute amounts varied just slightly between different transplants (**Fig. 6**). The major proportion of sGAG was released into the medium (**Fig. 7**). The amounts of sGAG did not correlate with the determined number of cells embedded in matrices and under cell culture conditions.

Discussion

Early treatment of localized cartilage lesions is the prerequisite for avoiding the onset of progressive cartilage degeneration and osteoarthritis. One option is the transplantation of cell suspensions of autologous chondrocytes at defect sites, which has proved to efficiently support reconstitution of joint functionality.[6] Nevertheless, this technique has some well-known drawbacks, such as the risks of leakage of cells from the implantation site, transplant hypertrophy, calcification, and delamination due to the deployment of the periosteum.[6-10] Many of those disadvantages can be circumvented by using autologous chondrocytes embedded and hence immobilized in matrices (matrix-coupled autologous chondrocytes).

In this study, we gathered parameters of the chondrocytic phenotype or differentiation of collagen-coupled autologous chondrocytes (CaRes®) at the time of transplantation. We found chondrocytes more or less equidistantly distributed within the scaffold as visualized by staining of histologic slices of various transplants. Marginal inhomogeneities in the spatial distribution of cells are most probably due to the processing of transplants for histology. Chondrocytes were spindle shaped rather than spheroidal or oval, the typical shape of chondrocytes in native cartilage. Cells seem to attach to single or only a few fibers of the loose collagen meshwork of transplants rather than being surrounded by the dense pericellular ECM characteristic for cartilage. As a consequence, they are forced to adopt the observed
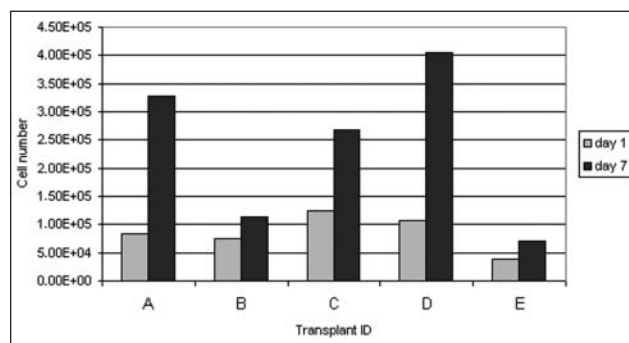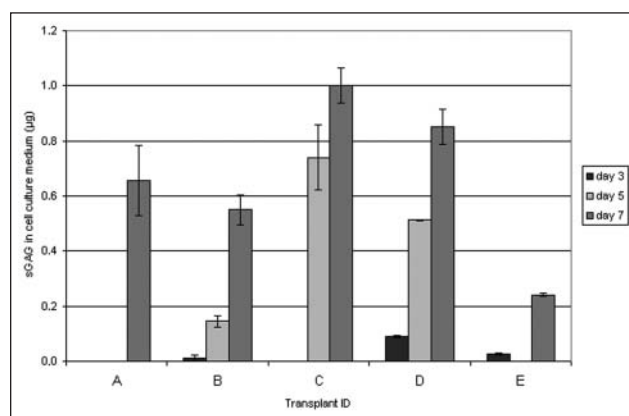

Figure 6. Amount of sulphated glycosaminoglycans released into the culture medium by chondrocytes isolated from 5 transplants (**A-E**).
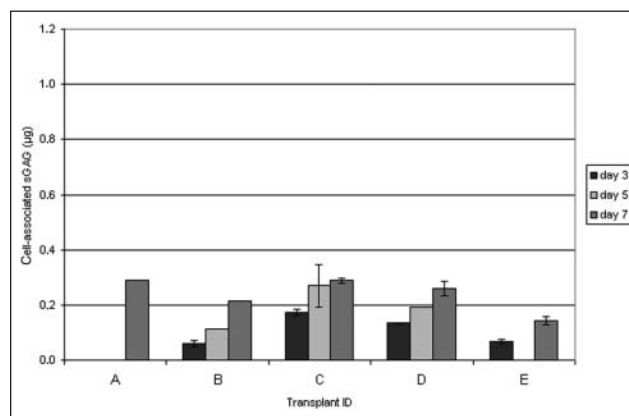

Figure 7. Amount of sulphated glycosaminoglycans associated with the cellular fraction of transplant-derived chondrocytes cultivated on standard conditions.

longitudinal morphology.[11] Although no cell clusters could be observed in histologic slices, proliferation took place as indicated by the increase of cell numbers during incubation after matrix embedment.[12,13] The lack of cell clusters might

be due to the detachment of cells after cytokinesis and their reattachment to collagen fibers, which comprise a rather loose meshwork in contrast to the dense ECM of the native tissue. The cell numbers of different transplants ranged from 5×10^4 to 3.5×10^5 cells/g transplant, and cells were viable to nearly 100% after isolation. For fabrication of cell-seeded matrices, chondrocytes were isolated from patients' cartilage biopsies and embedded into collagen I matrices without previous expansion in 2D culture. By that, dedifferentiation of chondrocytes during cultivation is restrained[14,15] or the expression of the chondrocytic phenotype even promoted.[12] Significant amounts of transplant-associated sGAG were found, indicating the chondrocyte-specific synthesis performance of cells in the course of time of cultivation. By contrast, collagen II expression was low in comparison with that of collagen I or even undetectable with the applied method for RNA isolation directly from paraffin-embedded specimens and consecutive RT-PCR. Because avoidance of 2D expansion as well as cultivation of chondrocytes in the collagen matrix favors the retainment of the chondrocytic phenotype, we suppose that the high collagen I/collagen II ratio and thereby obvious dedifferentiation is either a result of the 24-hour enzymatic digestion period or to the original constitution of cells used for embedment. These data, less affected aggrecan as well as significantly lowered collagen II expression, are consistent with previous findings concerning the influence of digestion with collagenase II on expression of aggrecan and collagen II.[16]

In addition, we observed that the amounts of sGAG of the various transplants varied markedly but did not correlate with the number of cells within the matrix. This result might be ascribed either to the different synthesis performance of cells or to the different retainment capacity of sGAG in the matrices. Variations of collagen concentrations that would influence the diffusion rate of proteoglycans and hence release from the matrix could be excluded because the manufacturing process of the investigated ready-to-use transplants is standardized to keep this parameter constant. To investigate if the different amounts of sGAG associated with the transplant were due to the secretion performances of cells or to their capability to retain sGAG at the cell surface, chondrocytes were isolated and further cultivated on standard cell culture conditions. Although care has to be taken with respect to drawing conclusions concerning the behavior of matrix-embedded chondrocytes, this approach proved to be useful in distinguishing between cell-associated and medium-released sGAG content. In addition, the presence of a high percentage of serum fosters the manifestation of interindividual differences in terms of proliferation.[17] We found significant differences with respect to proliferation and sGAG synthesis. Although total sGAG synthesis varied significantly, the fraction retained at the cell surface was marginal and similar in comparison with all investigated cells. This indicates interindividual variations of cells with respect to proliferation rate and secretion performance of sGAG but not their capability to retain newly synthesized sGAG at the cell surface.

In conclusion, we show that autologous chondrocytes embedded in collagen I matrix produce significant but variable amounts of sGAG. The high ratio of collagen I and collagen II in transplants might reflect the original constitution of chondrocytes from patients' biopsies. This is in accordance with reports stating high variability of chondrocytes with respect to the disease state of the joint, the age of the donor, and between individuals.[18-20] Nevertheless, follow-up studies proved the successful integration of collagen I/III matrix-coupled autologous chondrocytes,[21] and the use of transplants as investigated in this work has delivered promising results in a prospective clinical study.[22] However, further molecular characterization of chondrocytes for matrix-coupled autologous chondrocytes and the concomitant monitoring of patients' outcomes are needed to identify markers indicating regenerative potential.

Declaration of Conflicting Interests

Funding

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