The detached osteochondral fragment as a source of cells for autologous chondrocyte implantation (ACI) in the ankle joint

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Summary

Objective: Autologous chondrocyte implantation (ACI) has been successfully used for the treatment of osteochondral lesions of the talus. One of the main problems of this surgical strategy is related to the harvesting of the cartilage slice from a healthy knee. The aim of this study was to examine the capacity of chondrocytes harvested from a detached osteochondral fragment to proliferate and to serve as a source of viable cells for ACI in the repair of ankle cartilage defects.

Methods: Detached osteochondral fragments harvested from the ankle joint of 20 patients with osteochondral lesions of the talus served as the source of human articular cartilage specimens. All of the osteochondral lesions were chronic and of traumatic origin. In all cases, the fragments were utilized to evaluate the viability and proliferation of the cells, the histological appearance of the cartilage tissue and the expression of specific cartilage markers by real-time polymerase chain reaction (PCR). In the 16 patients scheduled for ACI, the expanded chondrocytes were used for chondrocyte implantation. In the other 4 patients, with lesion size <1.5 cm², microfractures were created during the initial arthroscopic step. As a control group, 7 patients with comparable osteochondral lesions underwent the same surgery, but received chondrocytes harvested from the ipsilateral knee.

Results: According to the American Orthopaedic Foot and Ankle Scoring (AOFAS) system, patients in the experimental group had a preoperative score of 54.2 ± 16 points and a postoperative one of 89 ± 9.6 points after a minimum follow-up time of 12 months (P < 0.0005). The control group of patients had a preoperative score of 54.6 ± 11.7 points and a postoperative one of 90.2 ± 9.7 points at a minimum follow-up time of 12 months (P < 0.0005). The clinical results of the two groups did not differ significantly from each other. Chondrocytes isolated from the detached fragments were highly viable, phenotypically stable, proliferated in culture and redifferentiated when grown within the three-dimensional scaffold used for ACI. The morphological and molecular characteristics of the cartilage samples obtained from the detached osteochondral fragments were similar to those of healthy hyaline articular cartilage.

Conclusions: The good results achieved with this strategy indicate that cells derived from the lesioned area may be useful in the treatment of osteochondral defects of the talus.

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Key words: Osteochondral lesion, Autologous chondrocyte implantation, Osteochondral fragment.

Introduction

Osteochondral lesions of the talus occur in approximately 6.5% of all ankle sprains, and in most cases following inversion or eversion injuries of the ankle in young and active people practicing sports. The intrinsic inability of articular cartilage to heal has been widely documented and this frequently necessitates surgical treatment.

The repair tissue produced after implementing surgical techniques such as chondral shaving or microfracturing bears no resemblance to the original hyaline cartilage and has poor mechanical properties.

Brittberg et al. developed the autologous chondrocyte implantation (ACI) technique, which permits the repair of osteochondral lesions of the knee with a high percentage of hyaline-like cartilage tissue. Consequently, the ACI technique has been applied also to other joints, with satisfactory results particularly in the ankle. After a follow-up time of more than 36 months in the latter, the clinical symptoms were improved, and the histological analysis of biopsy specimens revealed the formation of a repair cartilage with typical hyaline-like features. Although no complications have been reported to date, one of the main problems of this surgical strategy is related to the harvesting of the cartilage slice from a healthy knee.

In addition, it has been shown that knee cartilage differs in composition from ankle cartilage, having a lower level of proteoglycan synthesis and less active chondrocytes. These different characteristics might explain why the ankle is less affected by progressive osteoarthritis than the knee.

In order to avoid damage to a healthy knee due to the harvesting of cartilage, and to utilize cartilage with the appropriate characteristics for implantation in an ankle joint, it may be reasonable to use the detached osteochondral fragments for chondrocyte cultures.
The aim of this study was to examine the ability of the chondrocytes harvested from detached osteochondral fragments, to proliferate and to serve as a viable source of cells for ACI in the repair of ankle cartilage defects.

Patients and methods

Patients

Human articular cartilage specimens were obtained from the detached osteochondral fragments harvested from the talus of 20 patients with osteochondral lesions of this joint. The patients (11 males and 9 females) had a mean age of 30.5 ± 8 years. All of the lesions were of traumatic origin and the mean time elapsing between the traumatic event and surgery was 10 months (range: 6–20 months).

Preoperatively, all of the patients were evaluated radiographically and clinically using the American Orthopaedic Foot and Ankle Scoring (AOFAS) system. Amongst the inclusion criteria was a preoperative magnetic resonance imaging (MRI) demonstrating an osteochondral lesion and a detached osteochondral fragment. In all of the patients scheduled for surgery, the fragment was definable, still in situ with breached articular cartilage, and classified as stage III according to the DiPaola arthroscopic and MRI system. As a control group, seven patients (mean age: 26.8 ± 9 years) with similar characteristics and who were affected by comparable osteochondral lesions underwent the same surgery, but received chondrocytes harvested from the ipsilateral knee.

The study design was approved by the Ethics Committee at our Institution and informed consent was obtained from all of the patients enrolled.

Surgical Technique

In each of the 20 cases, an initial arthroscopy of the ankle was performed, which permitted fragment removal, a mild osteochondral shaving, and an accurate measurement of the lesion size. According to the guidelines recently proposed by the senior author (SG), the 16 patients with a lesion size > 1.5 cm² were scheduled for ACI. In the remaining 4 cases, with a lesion size < 1.5 cm², microfracturing was performed during the initial arthroscopy.

Two parts of the detached fragments obtained from the ankles were used. One sample was utilized for histology and immunohistochemistry. The other sample was processed for chondrocyte isolation, and the cells thereby obtained were used for real-time polymerase chain reaction (PCR) and the PicoGreen® proliferation tests. As controls, two biopsy samples of healthy cartilage were obtained from the talus of two different male multiorgan donors, aged 20 and 37 years, who did not have a known history of arthritis or related pathologies.

In the 16 patients scheduled for ACI, chondrocytes were expanded from one of the fragments. These were implanted 30 days after harvesting using Hyalograft-C (Fab Advanced Biopolymers, Abano Terme, Padova, Italy).

In the control group of 7 patients, cartilage was harvested arthroscopically from the ipsilateral knee for cell culturing. As in the experimental group, chondrocytes were expanded and then implanted 30 days after harvesting using Hyalograft-C.

In both groups, the second arthroscopic step was performed using traditional anteromedial and anterolateral arthroscopic accesses. The lesion was accurately shaven and sized. Since none of the lesions had a depth > 5 mm, no cancellous bone grafting was undertaken in this series.

The chondrocytes were directly supported on an auto-adhesive hyaluronan patch, which was arthroscopically positioned over the lesion. Fibrin glue was used to improve the stability of the transplanted matrix.

Chondrocyte Isolation and Growth Within a Three-Dimensional Matrix

Chondrocytes were isolated enzymatically as previously reported. Fragments of the cartilaginous tissue were cultured in Dulbecco’s modified Eagle’s medium [DMEM, Life Technologies, Paisley Scotland containing 25 mM HEPES (Sigma), penicillin [10,000 units/ml (Gibco)], streptomycin [10,000 µg/ml (Gibco)], and gentamycin [50 µg/ml (Biological Industries, Kibbutz Bet Haemek, Israel)]. Chondrocytes were isolated by sequential enzymatic digestions at 37°C; 30 min with 0.1% hyaluronidase (Sigma, St Louis, MO, USA), 1 h with 0.5% pronase (Sigma) and 1 h with 0.2% collagenase (Sigma). The isolated chondrocytes were filtered successively through sterile nylon meshes with pore diameters of 100 µm and 70 µm to remove cell and matrix debris. The filtrate was then centrifuged at 1800 rpm for 10 min. The pellet was washed twice with DMEM supplemented with 10% fetal calf serum (Biological Industries, Kibbutz Bet Haemek, Israel).

The cell number and viability were assessed by staining with eosin. The cells were cultured under conventional monolayer conditions at 37°C in a humidified atmosphere containing 5% CO2 for about 3 weeks (3–4 passages). The medium was changed twice a week.

To evaluate the chondrocyte redifferentiation process, cells were seeded within the same hyaluronan-based three-dimensional biomaterial (Hyaff®-11, Fidia) that was used for ACI. To this end, 1 x 10⁶ confluent cells were seeded within a 1 x 1 cm, 2-mm thick scaffold in a Petri dish (Becton Dickinson, Plymouth, UK) containing 150 µl of culture medium. The cells were allowed to adhere for 8 h at 37°C and before adding 2 ml of medium. The medium was changed twice a week. The constructs were analyzed after 1, 7, 14 and 21 days. Prior to seeding within the biomaterial, 1 x 10⁶ cells were pelleted and utilized as a dedifferentiated control (dedifs).

Proliferation Test

Cell proliferation rates were determined in triplicate at different experimental times (1, 7, 14, 21 and 28 days) using the PicoGreen® dsDNA Quantitation kit (Molecular Probes, Eugene, OR, USA) for the double-stranded DNA assay. After collecting the supernatants, the adherent cells were lysed with 100 µl of lysis buffer. PicoGreen® working solution (100 µl) was then added to each well. After incubating for 5 min at ambient temperature, the fluorescence of each sample was determined using a Spectramax Gemini dual-scanning microplate spectrofluorimeter (Molecular Device, Sunnyvale, CA, USA) in the well-scan mode, at an excitation wavelength of 480 nm, an emission wavelength of 540 nm and a cut-off wavelength of 515 nm. Fluorescence readings were converted to specific values using a DNA standard curve.

Quantification of the Expression Cartilage-Specific Molecules by Real-Time PCR

5 x 10⁵ cells that had been cultured in monolayers immediately after isolation were pelleted and then lysed in...
0.5 ml of RNAWIZ™ reagent (Ambion, Austin, TX, USA). Hyaff®-11 scaffolds containing seeded chondrocytes were collected at the different experimental times, placed in Microcon-100 filtration devices and then centrifuged at 1500 × g for 5 min. The supernatant was discarded and the cells lysed directly within the culture scaffolds by adding 0.5 ml of RNAWIZ™ reagent. Total RNA was isolated from the samples using the single-step, guanidinium thiocyanate—phenol—chloroform method.

Complementary DNA was synthesized from 1 µg of total RNA per sample at 42 °C for 45 min using Moloney Murine Leukemia Virus Reverse Transcriptase (Perkin Elmer, Norwalk, CT, USA) and oligo-(dT) priming.

Real-time PCR was conducted in a LightCycler instrument (Roche Molecular Biochemicals, Mannheim, Germany) using the QuantiTect™ SYBR® Green PCR kit (Qiagen, GmbH, Germany) and the following protocol: initial activation of HotStarTaq™ DNA polymerase at 94°C for 15 min, followed by 45 cycles of 15 s at 94°C, 20 s at 56–60°C and 10 s at 72°C. The increase in the PCR product achieved after each amplification cycle was monitored as an increase in the fluorescence generated by the binding of the SYBR Green I dye to dsDNA. The threshold cycle (Ct) values (i.e., the cycle number required, for the detected fluorescence to attain a threshold value in the range of the exponential amplification) were determined for each sample; the specificity of the amplicons was confirmed by a melting curve analysis and by agarose gel electrophoresis. The sequences of the PCR primers for aggrecan and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an internal control, were in accordance with reference standards.15,16 PCR primers for type-I and type-II collagen were designed using PRIMER 3 software.17 Specific primer pairs, PCR product length, annealing temperatures and the relevant references are given in Table I.

Real-time PCR data were analyzed using the “comparative Ct method” described by Livak and Schmittgen. As a relative quantification method, it relates the PCR signal of the target transcript in a group (or in different groups) to that of a reference control group using the formula 2−ΔΔCt. The ΔCt value represents, the mean difference between the Ct for each target gene (aggrecan, type-I and type-II collagen) and that for the internal control gene (GAPDH). The ΔΔCt value for each specific gene represents the difference between the ΔCt for the different samples and the control group. By definition ΔΔCt = 0 and 2ΔΔCt = 1 for each control sample. Data pertaining to freshly isolated chondrocytes from patients with osteochondritis dissecans referred to those for cells obtained from the healthy cartilage of a multiorgan donor. Data obtained from chondrocytes of patients with osteochondritis dissecans and grown within Hyaff®-11 scaffolds for 1, 7, 14, and 21 days were referred to those for cells previously expanded in monolayer cultures.

Using this method, the data are presented as differences in gene expression (relative gene expression) normalized to an endogenous reference gene and relative to a control group.

HISTOLOGICAL ANALYSIS

Formalin-fixed samples were decalcified with Formical-2000 (Decal Chemical, Congers, NY, USA) for 2 h at ambient temperature. They were then dehydrated in a graded series of ethanol and embedded in paraffin. Sections of 4-µm-thick were prepared from the cartilage specimens, and the slides were stored at ambient temperature until required for analysis. Healthy human articular cartilage obtained from a multiorgan donor was used as a control.

Tissue sections were stained either with 0.001% Fast Green and 0.1% Safranin-O (Sigma) or with 1% Alcian Blue 8GX (Sigma).

IMMUNOHISTOCHEMISTRY

Immunohistochemical staining was performed using antibodies against human type-I collagen (Chemicon International, Temecula, CA, USA) and human type-II collagen (Chemicon). Paraffin-embedded sections were deparaffinized and rehydrated. To unmask the epitopes, samples were treated with 0.1% hyaluronidase (Sigma) in phosphate buffered saline (PBS) for 5 min at 37°C and then incubated 30 min at ambient temperature in 1× PBS containing either 5% normal rabbit serum (Dako, Carpenteria, CA, USA) or normal goat serum to prevent non-specific binding. The slides were then incubated with the primary antibodies against type-I collagen, or type-II collagen (diluted 1:20 in 0.04 M Trizma Base Saline (TBS), pH 7.6, containing 0.1% Triton X-100) for 1 h at ambient temperature. The slides were washed three times with 0.04 M TBS (pH 7.6) and then incubated with goat anti-mouse and anti-rabbit immunoglobulins labeled with dextran—alkaline phosphatase (Envision, Dako) for 30 min at ambient temperature. After three washes with 0.04 M TBS (pH 7.6) the reaction product was visualized using the New Fucsin Substrate System (Dako). Levamisole (5 mM, Sigma) was used to block endogenous alkaline phosphatase activity. The slides were counterstained with hematoxylin, mounted in glycerol gel and stored at 4°C until required for analysis. Negative controls were prepared by omitting the primary antibody. All of the samples were

<table>
<thead>
<tr>
<th>Table I</th>
<th>Real-time PCR primers description</th>
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<tr>
<td>RNA template</td>
<td>Primer sequences</td>
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<tr>
<td>Aggrecan</td>
<td>5′-TCG AGG ACA GCG AGG CC 3′-TCG AGG GTG TAG CGT GTA GAG A</td>
</tr>
<tr>
<td>Type-I collagen</td>
<td>5′-AGG TGC TGA TGG CTC TCC T 3′-GGA CCA CTT TCA CCC TTG T</td>
</tr>
<tr>
<td>Type-II collagen</td>
<td>5′-GAC AAT CTG GCT CCC AAC 3′-ACA GTG TTG CCC CGC TTA C</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-TGG TAT CGT GGA AGG ACT CAT GAC 5′-ATG CCA GTG AGC TTC CGG TTC AGC</td>
</tr>
</tbody>
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*Primer sequences were obtained from published references where indicated or designed using PRIMER 3 Software.
evaluated using a Zeiss Axioscope microscope (Carl Zeiss, Oberkochen, Germany).

**Results**

**CHONDROCYTE ISOLATION AND PROLIFERATION**

Chondrocytes isolated from the detached cartilage samples were highly viable (99.9%) and their proliferation rate was comparable to that of normal talar chondrocytes (Fig. 1).

**ANALYSIS OF THE EXPRESSION OF CARTILAGE-SPECIFIC MOLECULES BY REAL-TIME PCR**

Real-time PCR monitoring with the LightCycler and using a fluorescent dye permitted a rapid and sensitive detection of the mRNAs for extracellular matrix molecules. Relative gene expression levels calculated on the basis of $\Delta Ct$ values are shown in Fig. 2. The mRNA for collagen type-II was higher in the experimental samples than in the controls, whereas that both for collagen type-I and for aggrecan was lower. The relative gene expression levels calculated on the basis of $\Delta Ct$ values for cells expanded in monolayer cultures and then grown within the biomaterial for different experimental times are presented in Fig. 3. The expression of type-I collagen within the three-dimensional scaffold increased until day 7 and then decreased up to day 21 [Fig. 3(A)]. The mRNA for type-II collagen was not detected in monolayer cultures. After growing the cells within the biomaterial, it increased only after 14 days for [Fig. 3(B)]. The expression of aggrecan did not follow a regular trend. But after 21 days within the Hyaff$^{21}$-11 scaffolds, the level was higher than in monolayer cultures [Fig. 3(C)].

**HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSES**

The cartilage samples evaluated by staining with Safranin-O and Alcian Blue manifested a histological appearance that was typically hyaline. The extracellular matrix contained a high concentration of proteoglycans, as evidenced by its intense red coloration [Fig. 4(A,B)]. The immunohistochemical analysis for type-II collagen revealed a positive signal within both the extracellular matrix and many of the chondrocytes throughout the entire thickness of the cartilage [Fig. 5(A)]. The immunohistochemical analysis for type-I collagen was negative [Fig. 5(B)].

**CLINICAL**

Neither subjective nor objective complications were observed with the surgical procedure. The 16 experimental patients had a preoperative AOFAS of $54.2 \pm 16$ points. At a minimum postoperative follow-up of 12 months (range: $12-20$ months), the score was $89 \pm 9.6$ points ($P < 0.0005)$. All of the patients expressed their satisfaction with the results, and the five individuals who were followed up for the longest time were able to resume their previous sports activity level.

The control group of patients had a preoperative score of $54.6 \pm 11.7$ points and a postoperative one of $90.2 \pm 9.7$ points at a minimum follow-up time of 12 months ($P < 0.0005$). These patients were likewise satisfied with the results and they experienced no residual pain after the harvesting of cartilage from the ipsilateral knee. A comparison between the clinical results in each of the two groups revealed no significant difference.

**Discussion**

Acute displaced osteochondral lesions should be treated by immobilization or open reduction and internal fixation. Unfortunately, the diagnosis is frequently missed. Consequently, continuous motion across the fracture site leads to avascular necrosis of the subchondral bone, thereby rendering successful fragment fixation increasingly difficult. Because joint cartilage repairs but poorly, the damage may be irreversible. ACI in the ankle joint has proved to be a reliable and effective technique for repairing cartilage in young and active patients, yielding good clinical results and permitting the resumption of sports activities. For ACI, trauma at the donor site of the knee is low compared to that engendered by the harvesting of plugs for a mosaicplasty, and no complications have been reported to date. Nevertheless,
the need for an arthroscopy in a healthy knee is a disadvantage of the procedure. Sammarco et al. have reported that the removal from the ankle of even the small amount of cartilage needed for cell expansion may be potentially detrimental to the functioning of this joint.

With a view to avoiding the harvesting of cartilage from healthy non-weight bearing areas, Chaipinyo et al. evaluated the proliferative capacity of chondrocytes derived from debrided damaged areas of the knee. They found that these fragments could provide sufficient cells for ACI. Since interference with a healthy knee should be avoided in surgical procedures relating to the ankle joint, the possibility of using detached osteochondral fragments as a convenient source of cells for ACI is worthy of consideration.

In our patients, the osteochondral fragments were removed from the affected ankle during an initial arthroscopic step, thereby permitting a direct intraoperative evaluation of the lesions.

At a postoperative follow-up time of 12 months, the clinical results in 16 patients were rated as good to excellent.
and comparable to those previously achieved by ourselves using the traditional Genzyme technique. In this earlier study, an AOFAS of 91 points was achieved after a follow-up of 24 months. Furthermore, in the present study the clinical results of the patients who had received cells did not differ significantly from those in the control group who had received cells from the knee.

The viability of cells isolated from talus cartilage was very high, and the expanded chondrocytes were able to re-express their differentiated phenotype when seeded within the three-dimensional scaffold used for transplantation. The histochemical and immunohistochemical results also justify the use of detached fragments for ACI. The cartilage manifested typical hyaline features with respect to the orientation of its collagen fibers and the regular organization of its cells, with a tangential orientation in the superficial layer and a columnar one in the deep zones. Real-time PCR revealed the samples to be producing high levels of the mRNA for type-II collagen and low levels of that for type-I collagen.

These findings indicate that the cells were metabolically active and that they were therefore adequately supplied with nutrients and oxygen from the synovial fluid present in the detached cartilage fragments. Hence, these fragments may serve as a convenient and useful source of cells for ACI in the foot. Future comparative studies are needed to validate the efficacy of this technique.

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References


