Influence of cryopreservation, cultivation time and patient's age on gene expression in Hyalograft® C cartilage transplants

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Abstract

Purpose Our aim was to evaluate the impact of cryopreservation, cultivation time and patient's age on the expression of specific chondrogenic markers in Hyalograft® C transplants. **Methods** Gene expression of chondrocyte markers [collagen type I (*COL1A1*), *COL2A1*, aggrecan, versican, melanoma inhibitory activity (MIA) and interleukin (IL)-1 β] was analysed in cartilage biopsies ($n=17$) and Hyalograft® C transplant samples (non-cryopreserved=78, cryopreserved=13) by quantitative real-time polymerase chain reaction (PCR). Correlation analyses were performed to evaluate the influence of the above-named parameters on the level of gene expression. **Results** Cryopreservation of cells was found to decrease *COL2A1* and MIA significantly (4.6-fold, $p<0.01$ and 2-fold, $p<0.045$, respectively). The duration of cryopreservation had no further influence on the expression of these factors. No correlation was detected between cultivation time (75 ± 31 days) and the expression level of any gene. Cartilage transplants from older patients (>35 years) exhibited a significantly higher IL-1 β expression (3.7-fold, $p<0.039$) than transplants from younger patients (≤ 35 years). **Conclusions** Our data demonstrate that cryopreservation has a profound impact on chondrocyte metabolic activity by decreasing the expression of *COL2A1* and MIA in Hyalograft® C transplants, independent of the duration of cryopreservation.

Introduction

The treatment of cartilage lesions is challenging, since the self-regeneration potential of articular cartilage is very limited and common surgical techniques such as microfracture surgery often only lead to the formation of fibrous or fibrocartilaginous tissue [1]. Consequently, alternative treatment options have been developed using the chondrogenic potential of transplanted cells for the regeneration of the complex structure and properties of native cartilage [2]. Matrix-associated autologous chondrocyte transplantation (MACT) is the newest generation of this approach to tissue engineering [3]. In a two-step procedure chondrocytes are isolated from a small biopsy taken from a non-weight-bearing area of the joint, propagated in vitro, seeded onto a three-dimensional scaffold (which is then termed transplant) and implanted into the defect. During the in vitro proliferation chondrocytes undergo morphological and molecular biological changes. The chondrocytes become fibroblast-like and cease the production of collagen type II (COL2), aggrecan and others that account for the specific mechanical properties of cartilage in vivo [4, 5]. The use of three-dimensional scaffolds aims to reverse this dedifferentiation process [6] and simultaneously simplifies the implantation, since a periosteal flap for coverage of the defect is no longer necessary. Despite the wide variety of available scaffolds, only few are in clinical use today [7, 8]. For this study a hyaluronan fleece (Hyalograft® C) was used as scaffold. As previously described by our group [9], the hyaluronan scaffold forms a non-woven web of regular fibres with large inter-fibre distances (Fig. 1a, e, f). The morphology of the sparse quantity of cells located in the scaffold appears either spherical or elongated (Fig. 1b, c, d). For the hyaluronan scaffold, a good clinical and magnetic resonance

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Fig. 1 Characteristics of the hyaluronan scaffold. **a** Macroscopic view of the scaffold (*scale bar* 1 cm). **b** Staining with 4',6-diamidino-2-phenylindole (DAPI) showing the cell distribution (*scale bar* 500 μ m). **c, d** Histological Alcian blue staining illustrating the scaffold and cell morphology (*scale bars* 500 μ m and 50 μ m). **e, f** Scanning electron microscopy images revealing the scaffold architecture (*scale bars* 200 μ m and 10 μ m)

imaging (MRI) outcome up to seven years has been demonstrated in several studies [10–12].

Some weeks usually elapse between the first arthroscopic surgery and the implantation of the cell-graft hyaluronan scaffolds, in the course of which transplants are generated. In some cases, however, a much longer period is needed, e.g. when an early implantation is not possible for the patient for occupational reasons, the patient wants time to consider the operation or the first operation fails. To overcome this problem the chondrocytes are cryopreserved after isolation and thawed when the implantation date is foreseeable. Generally, the vitality of chondrocytes after thawing depends on the protocol and cryoprotectant agent used for the cryopreservation procedure. Best results were found with a combination of different cryoprotective agents [13, 14]. Although the technique of chondrocyte cryopreservation is commonly used and good viability of the cells is still given after thawing

[14], the influence of cryopreservation on the chondrogenic potential of the cells remains poorly investigated. The aim of this study was to analyse the influence of cryopreservation and other parameters, such as cultivation time and patient's age, on the expression of chondrogenic and inflammatory markers [*COL2A1*, *COL1A1*, aggrecan, versican, melanoma inhibitory activity (MIA) and interleukin (IL)-1 β] in cartilage transplants in a clinically relevant setting.

MIA or cartilage-derived retinoic acid-sensitive protein (CD-RAP) is a protein, mainly expressed in embryonic and adult cartilage tissue. It is described as a protein unable to induce differentiation of mesenchymal stem cells by itself, but able to modulate the action of bone morphogenetic protein (BMP)-2 and transforming growth factor (TGF)- β_3 supporting the chondrogenic phenotype [15]. In vitro it was identified as a specific marker for chondrocytic differentiation [16] and therefore chosen as chondrogenic marker in this study.

To our knowledge this is the first study to analyse a large number of cartilage transplant samples ($n=91$) in order to evaluate the influence of cryopreservation, cultivation time and patient's age.

Materials and methods

Cartilage and transplant specimens

Residuals of 91 Hyalograft® C autografts (Fidia Advanced Biomaterials, Abano Terme, Italy) were collected during surgery. Residuals of an initial biopsy were collected from 17 patients during arthroscopy. The graft is produced by the company as follows: The patient's chondrocytes are isolated from biopsies and multiplied in a monolayer culture. According to the company the maximum number of passages for two-dimensional cultivation is defined in the production process, which was not further specified. The cells are thereafter seeded onto the surface of the hyaluronan web at a density of $1 \times 10^6/\text{cm}^2$ and cultivated for at least two weeks. The cells are cultivated in foetal calf serum. On demand chondrocytes are cryopreserved by the company for transplantations in the remote future. In that case the isolated cells are cryopreserved and when the implantation date is fixed the cells are thawed and processed according to the procedure described above. The cells of 13 transplants had been cryopreserved before transplantation. The protocol used for cryopreservation is a corporate secret.

MACT patients were men and women 19–50 years of age with a defect size of greater than two cm^2 and no knee instability or malalignment (axis deviation under 5°). Patients were excluded from the study if they were obese (over 30 kg/m^2 body mass index), had totally or subtotally resected menisci (but not after partial meniscectomy), severe neurological disorders, metabolic arthritis, joint infections, tumours, psychiatric diseases, arthrofibrosis or autoimmune diseases or were pregnant. The required amount of cartilage tissue was gained from biopsies from the femoral intercondylar notch of the knee during arthroscopy and sent to the company for further processing. In order to reduce donor site morbidity only one biopsy was taken from the affected knee joint of each patient and used for analysis. Samples of the cartilage transplants were collected during surgery and stored in the transport medium at room temperature. Immediately after surgery the samples were brought to the lab for further processing. Biopsies were stored in RNeasy (Qiagen, Hilden, Germany) at -80°C and transplant samples in TRI Reagent (Sigma-Aldrich) at -80°C until RNA extraction. The operations were all done in the same hospital by one surgeon. Clinical studies were approved by the local Ethics Board and patients gave consent (148/2003, 738/2010).

Real-time polymerase chain reaction (PCR)

Lysis of the cells on the hyaluronan scaffold was performed by adding 1 ml of TRI Reagent™ (Sigma-Aldrich). RNA isolation was performed according to the standard protocol. For total RNA extraction from native cartilage the samples were frozen in liquid nitrogen and ground using a mortar and pestle. Further steps were performed using the RNeasy® plant mini kit (Qiagen, Hilden, Germany). RNA ($0.1\text{--}1 \mu\text{g}$) was reverse-transcribed to cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA).

The design of primers and probes has been described previously [7]. To avoid amplification of genomic DNA, the probes were placed at the junction of two exons. For IL-1 β the predeveloped TaqMan® assay (Applied Biosystems, Carlsbad, CA, USA) was used.

Real-time PCR amplification was performed and monitored using an ABI Prism® 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The master mix was based on the SensiMix™ Probe Kit (Quantace, London, UK). The thermal cycling conditions comprised the initial steps at 50°C for two minutes and at 95°C for ten minutes. Amplification of the cDNA products was performed with 40 PCR cycles, consisting of a denaturation step at 95°C for 15 seconds and an extension step at 60°C for one minute. Beta-2-microglobulin (*B2M*) was chosen as housekeeping gene, using the predeveloped TaqMan® assay (Applied Biosystems, Carlsbad, CA, USA) [17]. For a given amount of cDNA the range of the housekeeping gene was within ± 1.5 Ct of the median value; samples outside of this range were excluded from analysis. All cDNA samples were analysed in triplicate. Relative quantification of gene expression was performed using the comparative $\Delta\Delta\text{C}_\text{T}$ method in Excel 2007 (Microsoft, Redmond, WA, USA).

Statistical analysis

Statistical evaluation was performed using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean values $\pm$ standard deviation (SD). Variables were tested for normal distribution using the Kolmogorov-Smirnov test. Differences between biopsies and transplant samples were assessed by paired *t* tests. Statistical analyses of other groups were performed using unpaired *t* tests. For correlation analysis Pearson's correlation coefficient (*r*) was calculated. All statistical tests were considered statistically significant when *p* values were lower than 0.05.

Results

Residuals of 91 cartilage transplants were collected during surgery and analysed by real-time PCR. The distribution of

patients' age and sex is shown in Table 1. From the date of biopsy and date of implantation the so-called processing time of each scaffold was calculated. This processing time included the transport, the isolation and the cultivation of the cells until the designated implantation date. In non-cryopreserved samples the processing time can be equated with cultivation time, since transport and isolation were short and relatively constant parameters. The processing time in cryopreserved samples also included the time of cryopreservation. Due to the proportionally long duration of cryopreservation compared to cultivation time, the processing time of cryopreserved samples mainly corresponds to cryopreservation time.

The mean values of the processing time were 74.8 ± 31.2 days for non-cryopreserved samples and 348.7 ± 273.8 (min. 122, max. 974) days for cryopreserved samples. Cryopreserved samples were excluded from all tests except the comparison between gene expression in cryopreserved and non-cryopreserved samples.

In the non-cryopreserved samples no correlation was found between the cultivation time and the expression level of any analysed gene (*COL2A1*: $r = -0.212$, $p < 0.11$; *COL1A1*: $r = -0.183$, $p < 0.139$; aggrecan: $r = -0.130$, $p < 0.309$; versican: $r = -0.038$, $p < 0.77$; MIA: $r = -0.202$, $p < 0.09$; IL-1 β : $r = -0.007$, $p < 0.96$).

There was no significant difference in relative gene expression level of *COL1A1* and versican between the cartilage biopsies and the corresponding transplants at the time of transplantation ($n = 17$) (Fig. 2). However, dramatic declines in the relative expression of *COL2A1* (28.924-fold, $p < 0.001$), aggrecan (94-fold, $p < 0.001$) and MIA (12-fold, $p < 0.003$) were found in the transplant samples compared to the corresponding biopsy samples. Whereas IL-1 β was detected in all 17 correspondent transplant samples, IL-1 β was found only in four of 17 biopsies. Gene expression levels in the biopsies did not correlate with gene expression levels in the corresponding transplant samples.

Since a cartilage biopsy is sometimes taken for possible future transplantations, the cells are cryopreserved by the company until the date of the transplantation is scheduled. In 13 of 91 transplant samples the cells had been cryopreserved. A 4.6-fold lower *COL2A1* expression and 0.982-fold lower MIA expression were found in the cryopreserved samples ($p < 0.01$ and $p < 0.045$, respectively). No other significant differences in gene expression were detected (Fig. 3) and the length of the cryopreservation showed no correlation with any

analysed gene expression (*COL2A1*: $r = 0.035$, $p < 0.909$; *COL1A1*: $r = 0.243$, $p < 0.432$; aggrecan: $r = 0.267$, $p < 0.378$; versican: $r = 0.001$, $p < 0.999$; MIA: $r = 0.393$, $p < 0.184$; IL-1 β : $r = -0.508$, $p < 0.092$).

To analyse the influence of age on gene expression in the transplant samples, patients were divided into two groups: ≤ 35 years ($n = 42$) and > 35 years ($n = 32$). Cryopreserved samples were excluded. Cartilage transplants in the > 35 years group had a 3.7-fold higher IL-1 β expression ($p < 0.039$) compared to the ≤ 35 years group (Fig. 4). Also *COL2A1* and MIA expression showed a trend to be higher expressed (5.5-fold and 2.5-fold, respectively) in the older group, although it did not reach statistical significance ($p < 0.073$ and $p < 0.069$, respectively).

Discussion

The main findings of this study were: (1) cryopreservation reduced the production of COL2 and MIA in transplant samples; (2) gene expression did not depend on the time of cultivation or cryopreservation in transplant samples; and (3) older patients exhibited a higher IL-1 β expression in transplant samples than younger ones.

Cryopreservation is commonly used for the long-term storage of many cell types. The fundamental requirement of all preservation methods is to maintain the viability and phenotype of the cell or tissue during storage. While cryopreservation of whole cartilage tissue causes irreversible damage to chondrocytes [18, 19], isolated chondrocytes can be cryopreserved without major loss of viability [14]. However, if cryopreservation of chondrocytes is to be used clinically, it must be established that the chondrocytic phenotype of the cells is maintained during storage. Only a few studies have been published that study the affect of cryopreservation on chondrocyte phenotype, with somewhat conflicting results. It has been reported that total aggrecan synthesis was not affected by cryopreservation of chondrocytes [20]. Further, COL2 production has also been demonstrated in cryopreserved chondrocytes [21]. However, whether the level of COL2 production by chondrocytes is decreased by cryopreservation is still debated [22, 23]. Our aim was to analyse the effect of cryopreservation on the chondrogenic potential of chondrocytes used clinically for MACT.

Table 1 Descriptive statistics

	Total	Non-cryopreserved	Cryopreserved
<i>n</i>	91	78	13
Age at biopsy (years)	33.9 ± 8.9	33.3 ± 8.4	37.2 ± 11.3
Male/female	61/30	51/27	10/3
Processing time (days)		74.8 ± 31.2	348.7 ± 273.8

Fig. 2 Gene expression comparison of cartilage biopsies with the corresponding cartilage transplant samples. Gene expression was normalised to *B2M*. Relative mRNA levels were normalised to native cartilage (error bars = SD). For *COL2A1/COL1A1* and aggrecan/versican the ratio of the

two genes were calculated by the division of *COL2A1* expression by *COL1A1* expression and aggrecan expression by versican expression, respectively

Our data indicated that in MACT samples seeded with cryopreserved chondrocytes there was a significant reduction in the relative gene expression of *COL2A1* (4.6-fold) and MIA (2-fold), but not aggrecan. However, the duration of cryopreservation had no further effect on *COL2A1* and MIA gene expression or the gene expression of any of the other genes studied. Therefore, it appears that the process of freezing and/or thawing the cells is the primary factor affecting the expression of the genes analysed in this study. Since COL2 production is essential for development and maintenance of cartilage mechanical properties and tissue integrity, a persistent loss of COL2 production could negatively affect tissue formation after MACT. It is not known if the decrease in *COL2A1* gene expression is continued after in vivo transplantation. While there is evidence that the replacement

tissue formed by cryopreserved chondrocytes after in vivo transplantation results in a poorer quality tissue [19], further study is required to determine if the use of cryopreserved chondrocytes results in a decrease in the long-term success rate of the MACT procedure clinically.

It is known that chondrocytes dedifferentiate when they are proliferated in monolayer cultures. The expression of cartilage-specific genes decreases in favour of cartilage-unspecific genes over time. In our transplant samples, however, we found neither a positive nor a negative correlation between the total cultivation time and the expression of any analysed gene. This may arise from the long cultivation time (75 ± 31 days), where the chondrocytes are more or less completely dedifferentiated, or from a compensatory effect of the three-dimensional cultivation on the scaffold.

Fig. 3 Gene expression comparison of cryopreserved and non-cryopreserved cartilage transplant samples. Gene expression was normalised to *B2M*. Relative mRNA levels were normalised to non-cryopreserved samples (error bars = SD)

Fig. 4 Differences in IL-1β expression in patients ≤35 and >35 years of age. Gene expression was normalised to *B2M*. Relative mRNA levels were normalised to the ≤35 years group (error bars = SD)

In general, IL-1 β expression is associated with osteoarthritis and is known to induce cartilage degradation. The high IL-1 β expression found in transplant samples of older patients suggests that chondrocytes maintain the predisposition for these destructive processes even after proliferation and cultivation on a three-dimensional scaffold. This indicates that the increased expression of IL-1 β in higher age patients might have negative effects on the clinical outcome after MACT.

Limitations of this study are that it just included Hyalograft® C samples and that the exact culture conditions and the cryopreservation protocol of Hyalograft® C transplants are not known, since they are a corporate secret of Fidia. Therefore, the conclusion of this study can only be applied to this type of transplant. However, since cryopreservation is a common procedure in most companies manufacturing cartilage transplants, our findings may lead to further investigations in this specific field.

Conclusion

Our data demonstrate that cryopreservation has a profound impact on chondrocyte metabolic activity by decreasing *COL2A1* and *MIA* expression in Hyalograft® C transplants, independent of the duration of cryopreservation. This implies that cryopreservation for a short period of time should be considered very carefully, whereas the disadvantages demonstrated for cryopreservation for longer periods of time can readily be accepted when considering the benefits of cryopreservation. Since IL-1 β is known as a proinflammatory cytokine and induces osteoarthritis, the significantly increased IL-1 β expression in transplant samples of older patients indicates that this may negatively affect the outcome after MACT in these patients.

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Conflict of interest The authors declare that they have no conflict of interest.

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