Original Article

Re-differentiation of Human Articular Chondrocytes Is Not Enhanced by a Rotary Bioreactor System

(human articular chondrocytes / real-time PCR / rotary bioreactor system / hydrogel scaffold / tissue engineering)

A. BARLIČ AND N. KREGAR-VELIKONJA

Educell d.o.o., Ljubljana, Slovenia

Abstract. ACI is the most widely used cell-based surgical procedure for the repair of articular cartilage defects. The method is based on *in vitro* chondrocyte cultivation. Two different culture conditions, rotating-wall-vessel bioreactor and static culture, were assessed by their effect on the re-differentiation potential of human articular chondrocytes seeded into a hydrogel scaffold. Gene expression analysis of the tissue-engineered construct revealed no significant difference between the tested systems.

Introduction

The field of tissue engineering has been rapidly evolving during the last decade. One of the most promising achievements in the field consists in the *in vitro* engineering of functional tissue substitutes for subsequent *in vivo* transplantation. One such method, autologous chondrocyte implantation (ACI), was introduced for healing of focal articular cartilage defects in the knee (Brittberg et al., 1994). In order to obtain the required amount of cells for construct preparation, harvested chondrocytes are expanded *in vitro* in a monolayer culture system. The two-dimensional environment inherent to such cultures causes a loss of the typical hyaline-chondrocyte phenotype, as observed by change in cell morphology and decreased expression of collagen type II and aggrecan. On the other hand, expression of collagen type I and versican, major extracellular matrix molecules in fibrocartilage, is increased (Benya and Shaffer, 1982; Darling and Athanasiou, 2005; Barlič et al., 2008). When transferred to a non-adherent, three-dimensional (3D) culture system, cells re-acquire spherical form and hyaline-specific phenotype through a process known as re-differentiation (Benya and Shaffer, 1982; Bonaventure et al., 1994; Barlič et al., 2008). A variety of hydrogels are being employed as a scaffold material, providing 3D environment that organizes cells and generates stimuli to direct the formation of a desired tissue (Drury and Mooney, 2003). Numerous bioreactors for the 3D *in vitro* growth of chondrocytes are currently available (Koch and Gorti, 2002). Rotating-wall-vessel (RWV) bioreactors have been shown to stimulate chondrogenesis, based on their efficient mass transfer properties and potential hydrodynamic stimulation (Baker and Goodwin, 1997). The objective of the present study was to compare the effect of two different culture conditions, rotary bioreactor system vs. static culture, on re-differentiation of human articular cartilage assigned for ACI.

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Corresponding author: Ariana Barlič, Educell d.o.o., Šlajmerjeva 6, SI–1000 Ljubljana, Slovenia. Phone: +386 1 5438192; Fax: +386 1 5438203; e-mail: ariana.barlic@educell.si

Abbreviations: 3D – three-dimensional, ACI – autologous chondrocyte implantation, RWV – rotating wall vessel.

Material and Methods

Chondrocyte isolation and expansion

Human knee femoral cartilage samples (four) were obtained from the superfluous material of patients scheduled for the ACI procedure (approved by the National Medical Ethics Committee; code 91/05/05). Cell isolation, expansion, and preparation of alginate-agarose hydrogel constructs were described previously (Barlič et al., 2008). Briefly, cartilage samples were diced into small pieces and enzymaticaly digested. The number and viability of chondrocytes was assessed by the trypan blue exclusion test. Cells were seeded into culture flasks at a density of 3,000 cells/cm^2 and cultivated in a humidified incubator. When about 80% confluency was achieved, cells were harvested by trypsin solution and replated. First-passage chondrocytes (P1) were seeded into alginate-agarose hydrogel at a concentration of 10 x 10^6 cells/ml. Hydrogel-chondrocyte constructs had a shape of a discus (3 mm thick and 5 mm diameter). For each human sample, four discs were prepared (two for bioreactor culture and two for static culture conditions).

Bioreactor cultures

A high-aspect rotating vessel (Synthecon, Inc., Houston, TX), housing a disposable 10 ml rotating wall vessel, was used as a bioreactor device. The rotation rates were periodically adjusted (from 15 to 21 rpm) to maintain the hydrogel-chondrocyte constructs in the orbiting regime. For comparison, static cultures were also maintained in 10 ml of the same medium used in the bioreactor culture (DMEM/F-12 with 15% (v/v) human serum and 50 μg/ml gentamycin). The medium was changed twice per week, and samples were taken for gene expression analysis at t = 0, 2 and 4 weeks.

Real-time PCR

RNA isolation. Total RNA was isolated using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma, St. Luis, MO) according to the manufacturer's instructions. *cDNA synthesis*. cDNA was generated using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. *PCR amplification and analysis*. PCR reactions were performed and monitored using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). TaqMan® Universal PCR Master Mix and TaqMan® Gene Expression Assays on Demand™ for collagen type II (Col 2), collagen type I (Col 1), aggrecan (Agr) and versican (Ver) were used. Human *GAPD* (*GAPDH*) was chosen as a housekeeping gene. All chemicals were purchased from Applied Biosystems. cDNA samples were analysed in duplicates. The C_t value of the housekeeping gene was subtracted from the C_t value of the target sequence in order to obtain $\_C_t$ values. Amplification efficiencies, determined using standard curve runs, were in all cases within 0.98–1.03. The level of expression of each target gene relative to that of the housekeeping gene was calculated with the formula (2)abs($\_C_t$). Relative expression values indicate whether the gene of interest is expressed to a higher or lower extent than the housekeeping gene. Normalized expression values are reported in arbitrary units and can only be used for comparative analysis among samples.

Statistical analysis

Values are presented as mean ± standard deviation. Statistical significance ($P < 0.05$) was assessed by unpaired Student's *t*-test.

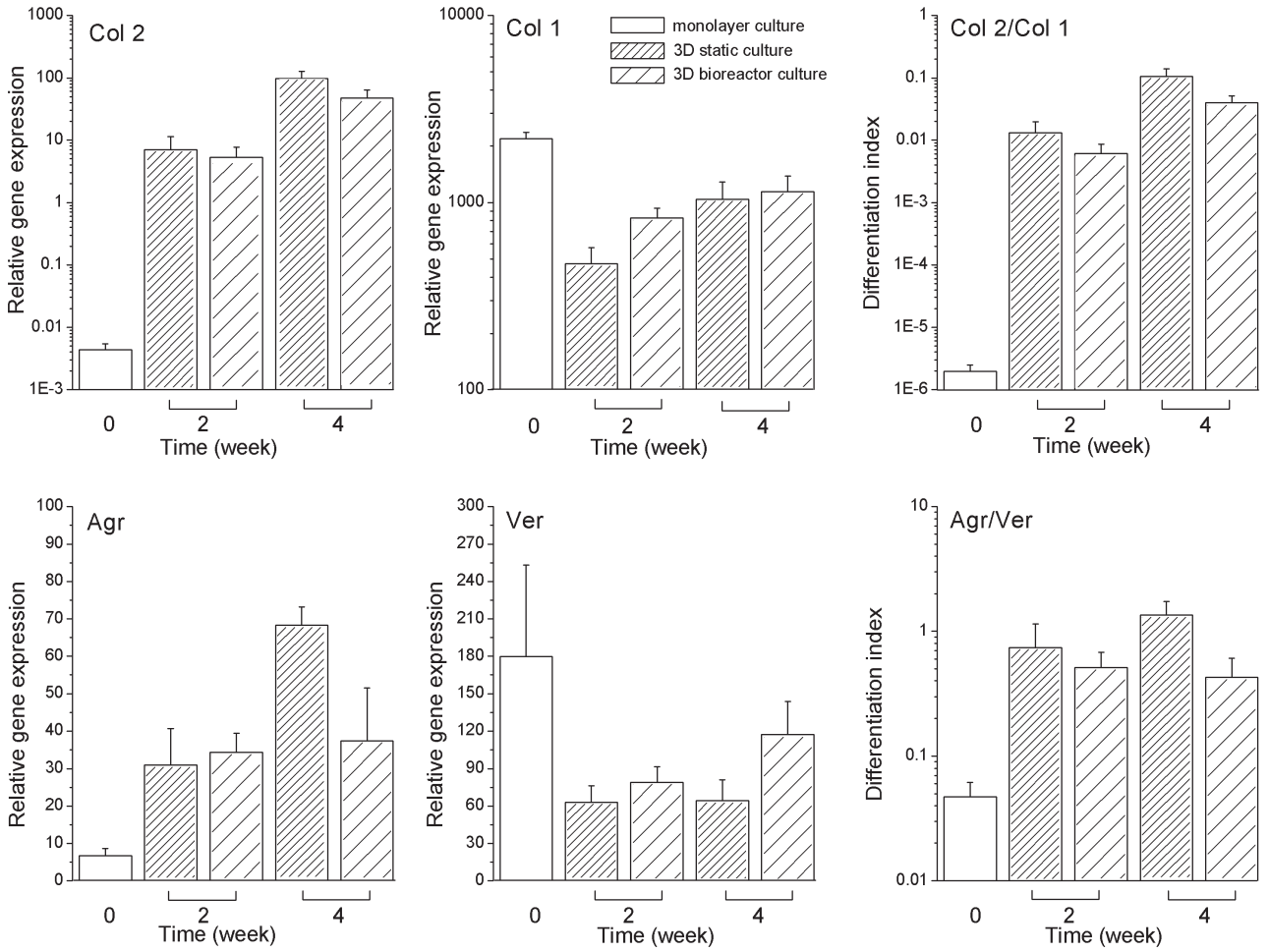

Fig. 1. Effect of culture conditions and cultivation time on relative gene expression values. Data represent mean ± SD for N = 4.

Results and Discussion

Successful ACI is based mainly on the quality of the implanted cells into the chondral lesion. The presented study is aimed to compare static culture conditions and rotating bioreactor system for culturing chondrocytes assigned for ACI. Gene expression levels for Col 2, Col 1, Agr and Ver were evaluated by the real-time PCR method and used to calculate the corresponding differentiation indices (Col 2/Col 1 and Agr/Ver). Differentiation indices are applied to describe the differentiation status of chondrocytes, where higher values signify more hyaline-like type of the cells (Martin et al., 2001). Col 2 and Agr, both specific markers for chondrocyte activity, have an important role in defining the mechanical integrity of articular cartilage. All relative gene expression values are presented in Fig. 1. As expected, expression of Col 2 and Agr was upregulated from monolayer first passage (P1) cells to second passage (P2) cells in the 3D static and bioreactor culture system, indicating successful re-differentiation of the chondrocytes after two weeks (Barlič et al., 2008). After four weeks of cultivation, re-differentiation values were even higher. When comparing static and bioreactor culture after two weeks, expression of Col 2 and Col 2/Col 1 differentiation index was slightly higher (approximately 2-fold) in static culture conditions. The difference was not statistically significant. The same phenomenon was observed after four weeks. Expression of Agr and Agr/Ver followed the same pattern at both time points. Statistically insignificant, slightly higher, expression in static culture as compared with bioreactor culture was observed. On the other hand, expression of Col 1 and Ver, markers for fibroblastic phenotype, was downregulated when the cells were seeded into the hydrogel. However, relative gene expression values were slightly higher in a bioreactor culture system than in static conditions, but the difference was not statistically significant. As has been suggested recently, bioreactor enhances cell proliferation and matrix synthesis of chondrocytes in alginate beads (Akmal et al., 2006). The tissue-engineered construct used in our study contained 10 x 10^6 cells/ml, a value close to the chondrocyte concentration in the native articular cartilage (Stockwell, 1971); therefore, the effect of bioreactor on the cell proliferation was not the focus of our study. Concerning the effect of bioreactor culture system on the chondrocyte re-differentiation process, no significant difference between the two tested systems was observed. According to other authors, use of the bioreactor culture did neither translate into increased matrix production nor improve the mechanical properties of chondrocytes seeded in agarose gel; therefore, higher cell densities are proposed to benefit from rotating bioreactor conditions (Hu and Athanasiou, 2005). In conclusion, our results suggest that cultivation of tissue-engineered constructs for cartilage repair in the rotary bioreactor does not out-perform static culture conditions.

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