Basics of isolation and cultivation of chondrocytes according to good laboratory practice

PRINCIPIOS EN EL AISLAMIENTO Y CULTIVO DE CONDROCITOS DE ACUERDO A BUENAS PRÁCTICAS DE LABORATORIO

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OBJECTIVES: THE OBJECTIVE OF THE PRESENT STUDY WAS TO DETERMINE IF CHONDROCYTES ISOLATED FROM HUMAN CARTILAGE OF FIVE ELDERLY PATIENTS (MEAN AGE 63) WITH OSTEOARTHRITIS (STAGE 3) MAINTAIN THEIR PROLIFERATION AND CHONDROGENIC POTENTIAL. ISOLATION AND CULTIVATION OF CHONDROCYTES WAS PERFORMED ACCORDING TO GOOD LABORATORY PRACTICE (GLP) STANDARDS.

METHODS: CHONDROCYTES WERE ISOLATED FROM CARTILAGE BIOPSY BY ENZYMATIC DIGESTION. CULTIVATION OF CELLS WAS PERFORMED IN A CONTROLLED ENVIRONMENT (CLEANROOM). PHENOTYPE CHARACTERIZATION OF CHONDROCYTES WAS ACHIEVED BY FLOW CYTOMETRY ANALYSIS.

RESULTS: THREE WEEKS AFTER CULTIVATION, POLYGONAL STRUCTURES TYPICAL OF CHONDROCYTES WERE OBSERVED, BUT SPINDLE/FIBROBLAST LIKE MORPHOLOGY WAS ALSO DETECTED IN CULTURED CELLS. FLOW CYTOMETRIC ANALYSIS SHOWED THAT CHONDROCYTES WERE POSITIVE FOR CD44 (98.35% ± 0.50), CD90 (97.15% ± 0.13) AFTER FIRST PASSAGE (P1) AND THE CELLS WERE NEGATIVE FOR HEMATOPOIETIC MARKER CD45 (0.21% ± 0.11).

CONCLUSIONS: HUMAN ARTICULAR CHONDROCYTES OBTAINED FROM FIVE ELDERLY PATIENTS WITH OSTEOARTHRITIS MAINTAINED A CHONDROCYTE PHENOTYPE AND COULD BE POTENTIALLY USED FOR AUTOLOGOUS IMPLANTATION. WE HAVE STANDARDIZED THE CONDITIONS FOR CULTIVATION ACCORDING TO GLP STANDARDS TO MINIMIZE THE RISK OF *IN VITRO* CELL CONTAMINATION.

KEYWORDS: AUTOLOGOUS IMPLANTATION, CARTILAGE, CLEANROOM, CHONDROCYTES.

PALABRAS CLAVE: IMPLANTACIÓN AUTÓLOGA, CARTÍLAGO, SALA BLANCA, CONDROCITOS.

Introduction

Cartilage tissue is composed of chondrocytes embedded within a dense extracellular matrix (ECM). It has poor autonomous regeneration capacity, mainly due to its avascular nature. Another factor contributing to poor regenerative capacity of articular cartilage (AC) is the restricted number of ECM producing cells. The percentage of highly specialized chondrocytes in cartilage tissue is only 1–3% (1). Chondrocytes are unable to migrate to a site of injury. They are able to synthesize fibrous repair tissue, but not sufficiently to fill small defects (<3 mm in diameter) with a cartilage-like matrix. Thus, many defects are not repaired and remain permanently (2). Cartilage was used as one of the first models for research of *in vitro* engineered tissues and has shown the earliest application for cell-based therapy mainly due to its cellular homogeneity and avascularity (3).

Chondrocytes within their natural environment actively synthesize and maintain their surrounding matrix. Mature chondrocytes have limited ability to proliferate and they are often mislabeled as dormant. In cell culture, human chondrocytes regain their ability to proliferate. Therefore, the basic premise behind Autologous Chondrocyte Implantation (ACI) is to overcome the inherent limitations of mature chondrocytes to effectively restore an injured articular surface.

In monolayer culture, these cells respond by undergoing rapid proliferation. Histologically, these chondrocytes reversibly dedifferentiate, assuming a fibroblastic appearance and expressing type I collagen as opposed to type II collagen normally seen in articular cartilage. Once removed from the monolayer culture and placed in suspension or returned to the articular cartilage environment, cells undergo a redifferentiation process into normal appearing chondrocytes and again produce type II collagen and proteoglycan aggregates (4).

In 1994 Brittberg *et al.* described the use of ACI in treating full-thickness AC defects from human knees (5). This was achieved in a two-stage procedure. Stage 1 involved arthroscopic biopsy of healthy AC and cultivation of the chondrocytes to produce between 5 and 10 million cells over a period of 4–6 weeks. Stage 2 involved debridement of the

osteochondral lesions and coverage by a periosteal flap followed by open implantation of these cells into the AC defect.

Isolation and cultivation of chondrocytes is a widely spread technique. It is required in ACI as a treatment for osteochondritis dissecans (6), osteoarthritis (7) and articular cartilage injuries produced by trauma (8). In general, it can be used in any articular cartilage injury, where other surgical procedures are not sufficient (4). *In vitro* chondrocyte manipulation is a crucial phase of autologous chondrocyte implantation. To minimize the risk of *in vitro* cell contamination, the manipulation must be performed in a controlled environment such as a cleanroom according to good laboratory practice (GLP). The GLP standards provide guidance on implementing GLP requirements critical for laboratory operations (9) (10).

In the current study, we have used basic methods for isolation and cultivation of chondrocytes from human articular cartilage according to GLP standards. Phenotype characterization of chondrocytes was performed by flow cytometry analysis.

Material and methods

Work in cleanroom according to GLP standards

Isolation and cultivation of chondrocytes were performed in the cleanroom in Associated Tissue Bank of the Faculty of Medicine of P. J. Šafárik University and L. Pasteur University Hospital in Košice, Slovakia. A cleanroom is a controlled environment where the concentration of airborne particles is controlled to specified limits (table 1). Contaminants generated by people, procedures, facilities and equipment were continually removed from the air. Air flow rates, direction, pressurization, temperature, humidity and specialized filtration were all tightly controlled. People and materials entered and exited the cleanroom through airlocks (material pass box), gowning rooms and they wore special clothing designed to trap contaminants that are naturally generated by the body and skin. Cells were handled inside biological safety cabinets and cultivated in the gas incubators (figure 1).

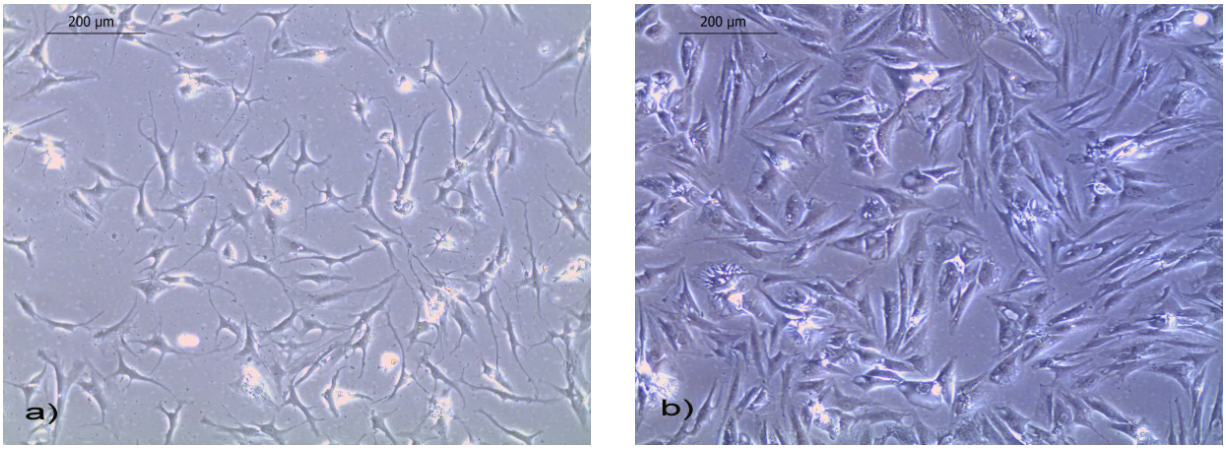
▲ **Figure 1**. *Cleanroom facilities for cell cultivation according to GLP standards.*

Isolation and cultivation of chondrocytes

Human cartilage tissue was harvested from the lateral femoral condyle of 5 patients (mean age: 63 years) undergoing total knee replacement surgery due to osteoarthritis (stage 3). Cartilage tissue was harvested in accordance with the ethical standards of L. Pasteur University Hospital commitee on human experimentation in Košice, Slovakia. Cartilage tissue was placed into the transport medium containing sterile

Class	Maximum particles/m³						FED STD 209E equivalent
	≥0.1 µm	≥0.2 µm	≥0.3 µm	≥0.5 µm	≥1 µm	≥5 µm	
ISO 1	10	2.37	1.02	0.35	0.083	0.0029	
ISO 2	100	23.7	10.2	3.5	0.83	0.029	
ISO 3	1,000	237	102	35	8.3	0.29	Class 1
ISO 4	10,000	2,370	1,020	352	83	2.9	Class 10
ISO 5	100,000	23,700	10,200	3,520	832	29	Class 100
ISO 6	1.0×10^6	237,000	102,000	35,200	8,320	293	Class 1,000
ISO 7	1.0×10^7	2.37×10^6	1,020,000	352,000	83,200	2,930	Class 10,000
ISO 8	1.0×10^8	2.37×10^7	1.02×10^7	3,520,000	832,000	29,300	Class 100,000
ISO 9	1.0×10^9	2.37×10^8	1.02×10^8	35,200,000	8,320,000	293,000	Room air

▲ **Table 1**. *Cleanroom classification according to the number and size of particles permitted per volume of air.*



▲ **Figure 2**. *Representative phase-contrast photomicrographs (magnification: x100) of cultured human (A) chondrocytes, 7 days of cultivation at Po (heterogenous cell population) (B) chondrocytes, 21 days of cultivation at Po (spindle and polygonal shaped cells).*

high-glucose Dulbecco's modified Eagle medium (DMEM; Invitrogen, GIBCO, USA) supplemented with 1% antibiotic/antimycotic solution (10,000 units/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL amphotericin B; Invitrogen, GIBCO, USA). Cartilage tissue was minced with a scalpel to small pieces (1x1x1 mm) and digested with 0,1% bacterial collagenase type II (Invitrogen, GIBCO, USA) in Ham's F-12 (Biochrom AG) for 20 h at 37 ºC in 95% air and 5% CO_2 humidified atmosphere. Cell suspension was filtered by a 40 µm nylon cell strainer (BD Falcon, Biosciences, Bedford, MA) to remove cell raft and matrix debris. The filtrate was then centrifuged at 150 x g for 7 min and the pellet was washed twice with DMEM (Invitrogen, GIBCO, USA). Isolated cells were suspended in cell culture medium containing Ham's F-12 (Biochrom AG), 10% fetal bovine serum (FBS; Invitrogen, GIBCO, USA), 1% antibiotic/antimycotic solution (10,000 units/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL amphotericin B; Invitrogen, GIBCO, USA) and 1% Insulin-Transferin-Selenium–A supplement (Invitrogen, GIBCO, USA). Chondrocytes were cultivated as a monolayer for expansion in 37 ºC humidified incubator with an atmosphere of 95% air and 5% CO_2. The medium was changed twice weekly. Confluent layers of chondrocytes were dissociated with 0.05% Trypsin-EDTA solution (Invitrogen, GIBCO®, USA) and the number and viability of cells was assessed by TC10™ Automated Cell Counter (Bio-Rad Laboratories).

Characterization of chondrocytes

Phenotype characterization of chondrocytes after first passage (P1) was performed by flow cytometry. After detaching the cells from the tissue culture flasks, cells were washed twice with phosphate buffered saline solution (PBS; Invitrogen, GIBCO, USA) supplemented with 2% FBS (Invitrogen, GIBCO, USA). Aliquots of 100,000 cells were incubated with mouse anti-human CD90-PE (Miltenyi Biotec Inc., USA), mouse anti-human CD44-PE (Miltenyi Biotec Inc., USA) and mouse anti-human CD45-FITC (Miltenyi Biotec Inc., USA) for 30 min in the dark. Flow cytometric analysis was performed with FACSCalibur flow cytometer (Becton Dickinson) and CellQuest software (Becton Dickinson). Chondrocytes were considered positive for a surface marker when the percentage of positive cells for that surface marker was ≥ to 95% and cells were considered negative for a surface marker when the percentage of positive cells for that surface marker was ≤ to 5%. The level of marker expression was calculated as the ratio between geometric mean fluorescence intensity of sample cells and that of the negative control.

Results

Chondrocytes isolation and morphology

Cells isolated from human cartilage after enzymatic digestion were seeded at 75 cm^2 tissue culture flask (T75) at density 35,000 cells/cm^2. The morphology of chondrocytes was observed under an inverted phase contrast light microscope (Leica DM IL). Non-adherent or low-adherent small round cells were also present in the primary culture after 4 days of cultivation. These cells were removed after the first medium change. Heterogeneous population of cells with different morphology was observed after 7 days of cultivation (figure 2a). After 21 days of cultivation, chondrocytes showed a more polygonal structure, even though few cells had spindle/fibroblast like morphology (figure 2b). Chondrocytes reached confluence within 3 to 4 weeks. The average number of chondrocytes obtained after trypsinization (P1) was 2.23 x 106 ± 0.5 per mL (table 2).

Sample	1	2	3	4	5	Average	SD
Number of cells (×10⁶)	2.53	1.62	2.35	1.81	2.82	2.23	0.50
Viability (%)	98.00	95.00	99.00	96.00	97.00	97.00	1.58
CD90 (%)	97.32	97.08	97.11	97.24	97.01	97.15	0.13
CD44 (%)	98.00	98.13	98.97	97.87	98.80	98.35	0.50
CD45 (%)	0.12	0.10	0.32	0.30	0.23	0.21	0.10

▲ **Table 2**. *Characterization of chondrocytes isolated from human cartilage of patients with osteoarthritis.*

Phenotype characterization of chondrocytes

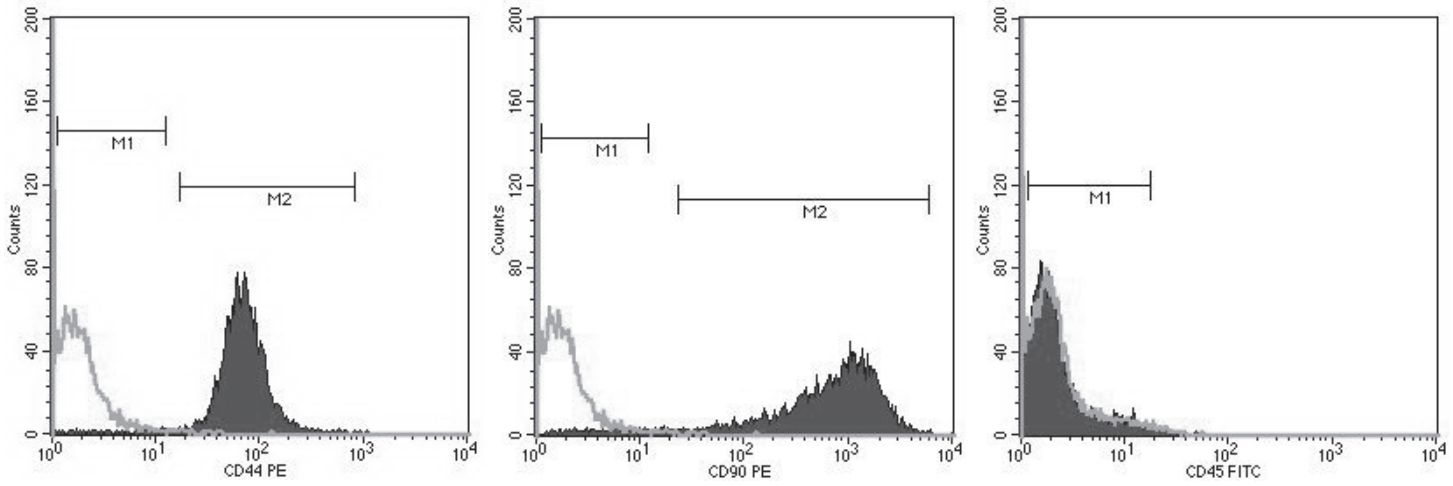
Flow cytometric analysis showed that chondrocytes were positive for CD44 (98.35% ± 0.50) and CD90 (97.15% ± 0.13) after first passage (P1) and cells were negative for hematopoietic marker CD45 (0.21% ± 0.11) (figure 3, table 2). Data are expressed as mean ± SD (n=5).

Discussion

The objective of chondrocyte manipulation suitable for autologous implantation is to obtain viable and phenotypically stable cells able to enhance repair processes in the damaged area of human cartilage. Cells carry out the balanced turnover of the extracellular matrix, which is necessary for the integrity of the extracellular cartilage. *In vitro* expansion of chondrocytes is possible via monolayer culture, whereby cells alter their morphology and metabolism in a process known as dedifferentiation (11). The importance of monolayer expansion of cells from small biopsies relies on its clinical implementation in repair strategies such as autologous chondrocyte transplantation (5).

The aim of the present study was to determine if chondrocytes isolated from human cartilage of five elderly patients with osteoarthritis (stage 3) maintain their proliferation and chondrogenic potential. It is known that upon digestion of the ECM from a cartilage biopsy and subsequent adhesion to the culturing surface, chondrocytes re-enter the cell cycle and proliferate. Three weeks after cultivation, polygonal structures typical of chondrocytes were observed, but spindle/fibroblast like morphology was also detected in culture. Samples obtained from aged patients, even in regions of "normal" appearing cartilage, are not metabolically normal and the chondrocyte phenotype is not stable *in vitro*, in particular in monolayer culture (12). Most data also suggest that the major phenotypic alterations are initially observed in surface areas of early-stage osteoarthritic cartilage, where chondrocytes express de novo abnormal, non-chondrocytic genes. In particular, they express the enzymes required for degrading the matrix that surrounds cells as well as many of the cytokines and growth factors relevant to turn on the catabolic processes within cartilage (13).

Immunophenotypic characterization of chondrocytes was performed by flow cytometry, which offers the possibility to assess and quantify a large number of epitopes on single cells within a short period of time. Immunophenotypic analysis of cells isolated from solid tissues through enzymatic digestions might be compromised due to the reduction or even total loss of surface molecules sensitive to enzymatic



▲ **Figure 3**. *Flow cytometric analysis of chondrocytes (P1). Cells were positive for CD44, CD90 and negative for CD45.*

treatment. Collagenase type II used in our study did not impair the detection of the CD90, CD44 and CD45 markers. We have confirmed the expression of the hyaluronan receptor CD44 and C 90 (Thy-1) on chondrocytes (1P) as previously reported (14, 15, 16). The hyaluronan receptor CD44 belongs to the polymorphic family of CD44 glycoproteins, which are involved in several cellular functions including adhesion to hyaluronan and collagen, and which is present in normal chondrocytes (15). It was previously reported that CD90 is also expressed in a minority of chondrocytes in normal articular cartilage (16). Up-regulation of markers on chondrocytes regarded as distinctive for mesenchymal stem cells (CD90 among others) during monolayer culture suggested that dedifferentiation leads to reversion to a primitive phenotype (17). Hematopoietic marker CD45 known as the leukocyte common antigen was not expressed by chondrocytes. The CD45 marker should not be present in normal chondrocytes but can appear in dedifferentiated ones (15).

It is important to enhance that cells used in this study were not applied in clinical practice and basic characterization of chondrocytes was the only aim of this study. A weakness of this study was the absence of a control group of healthy articular cartilage and from younger patients with osteoarthritis. This limitation was due to the lack of donors. Nonetheless, some researchers reported irreversible phenotypic changes between chondrocytes isolated from OA cartilage and chondrocytes from young healthy joints (18, 19, 20). Other reports indicate similar proliferation or differentiation potential of OA chondrocytes (21, 22).

Conclusion

Basic methods of *in vitro* isolation and cultivation of chondrocytes from OA cartilage were performed in this study. However, there were some limitations. More precise characterization of surface and intracellular markers of chondrocytes as well as comparison with a control group of young healthy patients should have been practiced. In conclusion, human articular chondrocytes obtained from elderly patients with osteoarthritis (stage 3) maintained a chondrocyte phenotype and could be potentially used for autologous implantation. We have standardized the conditions for cultivation to minimize the risk of *in vitro* cell contamination according to GLP standards since chondrocyte manipulation for autologous implantation requires standardized protocols to ensure that the cell product is therapeutically effective and safe.

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