MOLECULAR AND EXPERIMENTAL BIOLOGY IN MEDICINE

MESENCHYMAL STEM CELLS AND CHONDROCYTE CO-CULTURE PROMOTE CHONDROCYTE MATRIX PRODUCTION IN NORMOXIC CONDITIONS

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Abstract: Articular cartilage is tissue that shows no capacity for effective spontaneous regeneration. Tissue engineering, as a new important field of regenerative medicine, emerges as a potentially effective solution for the treatment of articular cartilage injuries and defects. The aim of this study was to evaluate chondrocyte oxygen level preference for three-dimensional (3D) *in vitro* chondrogenesis, and to test whether the presence of mesenchymal stem cells (MSCs) would influence oxygen level preference. In the experiment, we have set up two different cell cultures: only chondrocytes and a combination of chondrocytes and MSCs in a 2:1 ratio. Cells were grown in a chondrogenic medium, incorporated in a peptide hydrogel RADA, in either normoxic (20% O_2) or hypoxic (5% O_2) conditions. After 19 days, expression levels of two cartilage marker genes, *SOX9* and *ACAN*, were evaluated by real-time quantitative PCR. In conclusion, hypoxia significantly enhanced chondrogenesis but only in the case of chondrocytes, while co-culturing with MSCs slightly improved differentiation in normoxic conditions.

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Submitted: April, 2018
Accepted: July, 2018

Key words: mesenchymal stem cells, chondrocytes, cartilage, oxygen level, differentiation

INTRODUCTION

The primary goal of the tissue engineering (TE) approach is to produce tissue implants that structurally and functionally resemble native tissue that needs to be repaired. For successful engineering of each tissue, the right combination of cell type, scaffold and inductive signals (chemical and physical) must be used. Poor ability of self-repair, ineffectiveness of common treatment options, together with the growing problems of obesity and ageing of the general population, make articular cartilage an ideal candidate for the tissue engineering approach.

Articular cartilage is a form of hyaline cartilage, located in the diarthrodial (synovial) joints, and best characterized of cartilage subtypes[1], consisting of fluid (mostly water) and a solid, porous-permeable, dense extracellular matrix (ECM) which contributes to its biomechanical function.[2] Articular cartilage lacks blood vessels, lymphatic vessels, nerves, inflammatory cells and fibroblasts. It is composed of a single cell type, the chondrocyte.[1]

One of the basic challenges of cartilage TE is to identify an ideal cell source which should be easy to isolate, capable of expansion and production of cartilage-specific ECM molecules (collagen type II and aggrecan).[3] Chondrocytes are the most obvious choice since they are found in natural cartilage and have been extensively researched for cartilage repair.[4] Another potential source are fibroblasts which can be easily isolated, expanded and directed toward chondrogenic phenotype.[5] Recently, mesenchymal stem cells have been considered as a potential cell source for cartilage TE. These cells can be isolated from many different

tissues and show great capacity for differentiation *in vitro*.[6]

Scaffolds represent one of the key components of the TE approach. Scaffold materials facilitate the attachment, proliferation and differentiation of embedded cells, and function as a template which controls the geometry of newly formed tissue.[7] Hydrogels are materials consisting of a water-swollen, three-dimensional (3D) network which permits the attachment of molecules and cells. These scaffolds serve as a substitute for natural ECM to organize cells into 3D architecture and to present stimuli, which direct the growth and formation of the desired tissue.[8]

The third component of tissue engineering are different stimulating factors, which are able to induce, accelerate and enhance cartilage tissue formation. The first group are different growth factors like TGF-β, FGF, BMP and IGF, together with other soluble factors like insulin which affect cellular differentiation and cartilage tissue formation.[3] TGF-β1, 2 and 3 induce chondrogenesis and stimulate synthesis of ECM in both chondrocytes and MSCs. They trigger the signaling cascade that activates the expression of SOX9, a key transcription factor necessary for early chondrogenesis. SOX9 activates promoters of gene coding for collagen type II, aggrecan and other cartilage-building proteins.[9] Aggrecan is the major proteoglycan in the articular cartilage. This molecule is important for the proper functioning of articular cartilage because it provides a hydrated gel structure (via its interaction with hyaluronan and link protein) that endows the cartilage with load-bearing properties. FGF-2 preserves the chondrogenic potential of monolayer expanded chondrocytes and enhances proliferation.[3] In addition to growth factors, many protocols for chondrogenic differentiation also include ascorbic acid and dexamethasone (DEX). Ascorbic acid is required for collagen and proteoglycan synthesis. It has also been shown that it stimulates differentiation and proliferation of MSCs.[10] DEX is a synthetic glucocorticoid that has shown the ability to direct regulation of cartilage-specific genes in a culture of MSCs. Glucocorticoids usually act as inhibitors of chondrocyte cell growth and ECM synthesis, but reports suggest that specific DEX concentrations seem to have a synergistic effect with chondrogenic growth factors (especially TGF-β1), and thus contribute to chondrogenesis of MSCs.[11] Chondrogenic differentiation lasts 3 weeks *in vitro* and can be followed via chondrogenic markers at any time point between 7 and 21 days.

In the natural environment i.e. in the joint, chondrocytes are exposed to reduced oxygen tension (6-10% at the surface and only 2% in the deep zone) and elevated hydrostatic pressure. The mimicking of these conditions represents a new additional strategy for the improvement of *in vitro* cartilage formation.[3] Studies suggest that culturing of MSCs in hypoxic conditions (5% O_2) promotes the expression of chondrogenic transcription factors, including SOX transcription factors, synthesis of ECM and collagen type II production.[9] The transcription factor, hypoxia-inducible factor-Iα (HIF-Iα), is a crucial mediator of this cellular response to hypoxia.[12] Chondrocytes in the joint are also exposed to mechanical loading and forces which affect their metabolism and enable nutrient and oxygen exchange. Several studies have confirmed that the application of mechanical stimulation in vitro promotes differentiation and matrix production. A significant challenge which remains is to determine the optimal frequency, type and timing of mechanical loading on cultures for cartilage TE.[9, 13, 14]

In this study, we propose chondrocytes as tissue-forming cells. However, these cells are exposed to low oxygen levels in their natural habitat due to non-vascularized cartilage environment. Therefore, we assume that the cells will adjust better to hypoxic environment. As hypoxic conditions are technically more challenging, we hypothesized that the addition of MSCs in chondrocyte culture will result in better response to normoxic conditions and better chondrocyte differentiation. This research is an attempt to optimize conditions for cartilage tissue engineering. The specific goals of this research were: i) to establish a 3D culture system for cell growth in a self-assembling peptide hydrogel RADA using differentiation media composed of ascorbic acid-2-phosphate, insulin-transferrin-selenium, TGFβ-1 and DEX; ii) to determine and compare chondrogenic potential of chondrocytes and a combination of chondrocytes and MSCs in a 2:1 ratio; and iii) to demonstrate the effect of oxygen tension on the efficiency of chondrogenic differentiation. The level of differentiation was estimated after 19 days in culture, before the end-point of differentiation, according to the SOX9 and agreccan gene expression.

MATERIAL AND METHODS

Isolation and propagation of human articular chondrocytes and human mesenchymal stem cells (hMSCs) derived from bone marrow

Chondrocytes were isolated from articular cartilage of the knee obtained after knee surgery performed at University hospital "Sveti Duh" in Zagreb with the approval of the Ethical committee and after obtaining informed consent. Samples were minced followed by enzymatic digestion in 0.08% collagenase II in DMEM (Dulbecco's Modified Eagle's Medium, Lonza) overnight at 37°C. The cellular suspension was strained through a cell strainer (BD Falcon) and centrifuged at 300 x g for 5 min to obtain a cell pellet. The cells were grown in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% penicillin/streptomycin (pen/strep) (Lonza) and 1% L-glutamine (Gibco) at 37°C and 5% CO_2 with media changes every 3 days.

Human mesenchymal stem cells (hMSCs) derived from bone marrow were isolated at the Clinical center "Sestre Milosrdnice" in Zagreb with the approval of the Ethical committee and after obtaining informed consent, using the previously described method.[15] They were grown in low-glucose DMEM (Lonza) containing 10% FBS, 1% L-glutamine, 1% pen/strep

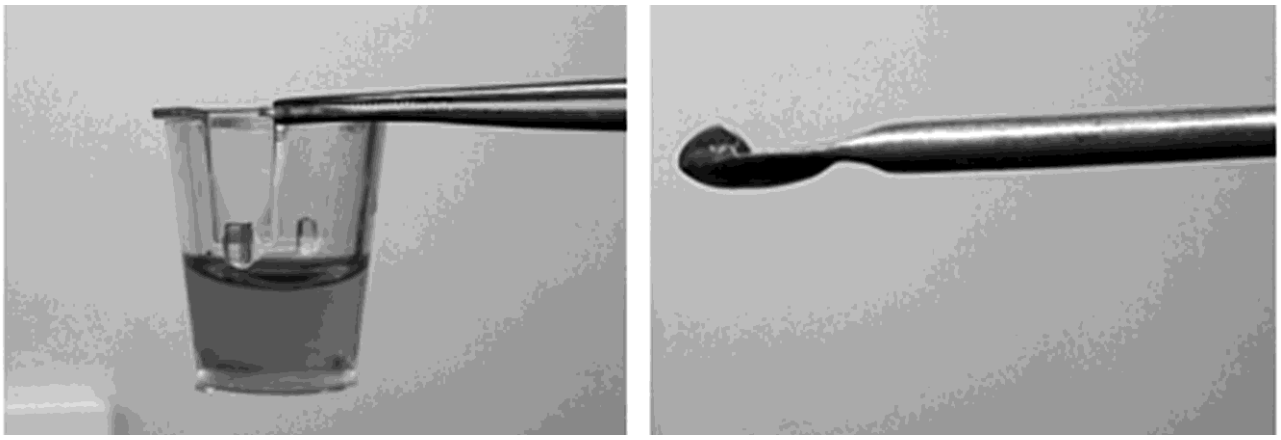

Figure 1. Tissue formed within peptide hydrogel after 19 days of chondrogenic differentiation. Transparent layer of peptide hydrogel is visible at the bottom of the insert (left) as well as on the spoon when taken out (right).

with the addition of FGF-2 (Gibco) in a concentration of 10 ng/mL.

Chondrogenic differentiation

The chondrogenic medium was composed of high-glucose DMEM supplemented with 10% FBS and 1% pen/strep with the addition of 40 µg/mL L-proline (Sigma), 50 µg/mL ascorbic acid-2-phosphate (Santa Cruz), 1 x insulin-transferrin-selenium (ITS) (Sigma), 10 ng/mL TGFβ-1 (Abcam) and 10^{-7} M dexamethasone (DEX) (Sigma).

Establishment of three-dimensional (3D) cell culture

Commercially available hydrogel (Corning®PuraMatrixTM Peptide) was used as a scaffold. A 3D cell culture was established by encapsulation of cells in the peptide hydrogel placed in cell culture inserts (0.4 µm pore size, Falcon) inserted in a corresponding 24-well plate. Cell pellets were resuspended in 1 mL of 10% sucrose to obtain a cellular suspension concentration 10^6 cells/mL. A suspension containing a combination of chondrocytes and hMSCs in a 2:1 ratio was prepared as a mixture of corresponding volumes of previously prepared suspensions of chondrocytes and hMSCs in 10% sucrose. To complete the gelation of hydrogel, plates were incubated for 1 h in an incubator, followed by media change.

Cell culture conditions for normoxia and hypoxia

One plate was placed in a standard cell culture incubator (Thermo Scientific Heraeus) in normoxic conditions (5% CO_2, 37ºC) and the other in Whitley H35 hypoxystation (20% CO_2, 37ºC) (Don Whitley Scientific). The chondrogenic medium was changed every 2-3 days during a period of 19 days.

Total RNA isolation and gene expression analysis by real-time quantitative PCR (RT-qPCR)

On the 19th day, the samples were taken out of the inserts and placed in 500 µL of TRIzol® reagent (Invitrogen), followed by homogenization with steel beads for 4 min at 15 Hz. The addition of chloroform and isopropanol was used for RNA isolation. Total RNA was treated with RNase free DNase I (BioLabs) according to the manufacturer's instructions. Purified RNA was then reverse transcribed in 20 µL reaction mixture containing 9.2 µL of DNase treated RNA sample, 4 µL of 25 mM $MgCl_2$ (Fermentas), 2 µL of 10x Buffer without $MgCl_2$ (Applied Biosystems), 2 µL of 10 mM dNTPs (Bio Basic Canada), 0.8 µL of Ribolock RNase inhibitor (Thermo Scientific, 40 U/µL), 1 µL of random hexamers (Invitrogen) and 1 µL of MuLV reverse transcriptase (Applied Biosystems). Reaction mixtures were incubated for 10 min at room temperature, 1 h at 42ºC, 5 min at 99ºC and 5 min at 5ºC. Samples were diluted to obtain cDNA in a final concentration of 20 ng/µL.

Relative expression of *SOX9* and *ACAN* genes was determined using commercially available primers (Sigma) and Power SYBR Green Mastermix (Applied Biosystems) on a 7500 Fast Real-Time PCR System machine (Applied Biosystems). The endogenous control was *β-actin*. Each reaction mixture contained 40 ng of cDNA. Results were analyzed using 7500 System Software v2.0.6. Fold changes in gene expression were calculated using the ΔΔCt method. Since qPCR reactions for samples from both hypoxic and normoxic conditions could not be performed on the same 96-well plate, the results for samples grown in hypoxic conditions were calibrated to the same reference sample to which results from normoxia were calibrated, thus enabling the comparison of results.

Statistical analysis

A descriptive statistical analysis of the data was performed using a one-way ANOVA analysis of

variance and Duncan test (Statistica 13.3). Differences were considered statistically significant at $P<0.05$. Data are presented as mean ± standard deviation (SD).

RESULTS

After 19 days of chondrogenic differentiation in normoxic and hypoxic conditions, scaffolds were taken out of the inserts (Figure 1) while the morphology of the formed tissue was observed under inverted light microscope (data not shown).

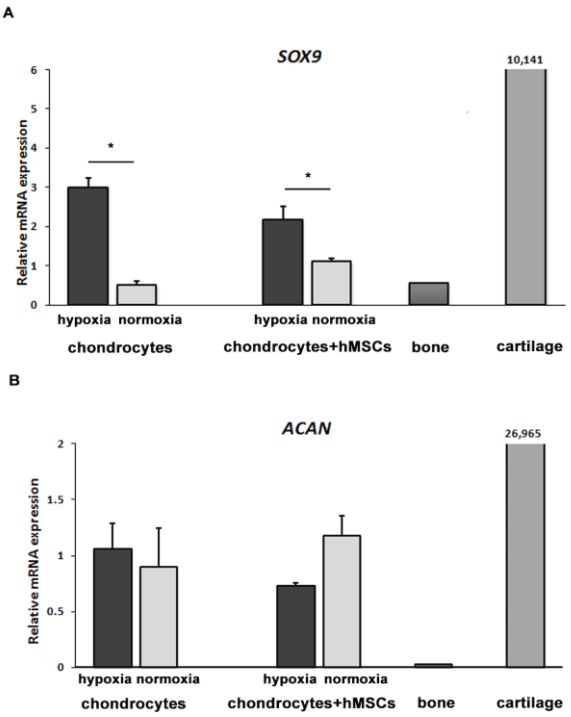

Figure 2. Relative mRNA expression of chondrogenic markers after chondrogenic differentiation of chondrocytes and chondrocytes in co-culture with hMSCs cultured in hypoxic and normoxic conditions. (A) Relative mRNA expression of *SOX9* and (B) *ACAN* was determined by real-time quantitative PCR on day 19. RNAs isolated from bone and native cartilage were used as a negative and positive control, respectively. The relative gene expression was analyzed by ΔΔ cycle threshold method and the values were normalized to *β-actin* expression. The results are presented as mean ± SD (n = 2). * P<0.05

In order to determine which cell type and culture conditions are the most beneficial for chondrogenesis and application in cartilage, expression levels of cartilage markers *SOX9* and *ACAN* were evaluated (Figure 2a and 2b). *SOX9* encodes for the cartilage-specific transcription factor SOX9, while *ACAN* encodes for aggrecan, the main proteoglycan of the extracellular matrix of the growth plate cartilage. The chondrogenic differentiation of chondrocytes and chondrocytes grown with hMSCs in a 2:1 ratio was held in a 3D culture system under normoxic or hypoxic conditions, respectively. RNAs isolated from bone and native cartilage were used as a negative and positive control, respectively. Relative expression of *SOX9* and *ACAN* in all samples was calibrated to the same reference sample – a combination of chondrocytes and hMSCs grown in normoxic conditions. As suspected, chondrocytes possessed the highest chondrogenic potential under hypoxic condition since relative expression levels of *SOX9* and *ACAN* were highest in hypoxia. Considering the influence of oxygen tension on chondrogenic differentiation of chondrocytes, hypoxic conditions proved to be more beneficial for chondrogenesis than normoxic conditions (Figure 2a). Relative expression levels of chondrogenic markers rise when chondrocytes are cultured together with hMSCs under normoxic conditions (Figure 2a and 2b). The results imply that adverse effects of normoxia on chondrogenesis of chondrocytes can be overcome by culturing them together with hMSCs. Therefore, if hypoxic conditions are not available, the combination of chondrocytes and MSCs in a 2:1 ratio is recommended for cartilage formation.

DISCUSSION

Cells were grown in a 3D system in cell inserts, encapsulated in a self-assembling peptide hydrogel $(RADA)_4$, a new class of scaffold widely investigated for the purposes of tissue engineering. Cells were grown in a chondrogenic medium for 19 days. In order to explore the effects of oxygen tension on chondrogenic differentiation, cells were grown in either hypoxic (5% O_2) or normoxic (20% O_2) conditions. After 19 days, samples were harvested and the expression of two cartilage marker genes *SOX9* and *ACAN* was analyzed using RT-qPCR.

According to the results of gene expression analysis, chondrocytes grown in hypoxic conditions showed the highest chondrogenic potential. Before the chondrogenic differentiation experiment, chondrocytes were expanded in a monolayer culture in order to obtain sufficient cell numbers for seeding in a peptide hydrogel. Several studies showed extensive changes in gene expression in chondrocytes upon passaging. This process mainly affects collagen II production and genes involved in signaling networks responsible for the maintenance of the chondrocyte's phenotype. Dedifferentiation represents the main obstacle to cartilage tissue engineering using chondrocytes as a cell source.[16] However, this process is reversible. Caron et al[17] investigated the effects of 2D and 3D culture systems on the redifferentiation of passaged chondrocytes. Three-dimensional cultures expressed cartilage-specific genes for collagen type II, aggrecan core protein and SOX9 transcription factor, while 2D did not. Furthermore, a reduced oxygen tension also proved to promote redifferentiation of passaged chondrocytes. Chondrocytes grown in hypoxic conditions (2-5% O_2) showed enhanced production of

ECM components (collagen type II, aggrecan, GAGs) and decreased production of collagen type I, collagen type X, matrix metalloproteinases and aggrecanases.[18–20] Therefore, based on these studies and the obtained results, it can be assumed that RADA encapsulation and reduced oxygen tension helped restore key differentiated phenotypic markers of passaged chondrocytes.

Co-culture of chondrocytes and hMSCs in a 2:1 ratio have been proved to have lower chondrogenic potential than chondrocytes. The influence of oxygen tension did not significantly influence chondrogenesis in co-culture, although samples grown in normoxia demonstrated higher expression levels of *ACAN* compared to samples grown in hypoxia. Cheng et al[21] demonstrated that co-culture of rabbit chondrocytes and rabbit MSCs at defined ratios can promote the expression of cartilaginous ECM. They determined the 2:1 ratio (chondrocytes:MSCs) to be optimal, since the expression of collagen type II and aggrecan in this group on day 21 was higher than in other groups containing individually cultured chondrocytes and MSCs or a co-culture of chondrocytes and MSCs in a 4:1, 1:1, 1:2 and 1:4 ratio. Neocartilage can develop through co-culturing of chondrocytes and hMSCs even in the absence of biomolecular factors such as serum and exogenous growth factors. It is hypothesized that chondrocytes promote chondrogenesis of MSCs via paracrine regulation since they produce and secrete a variety of protein molecules including TGF-β, IGF-1, FGF-2, BMP-2, and many other inductive factors that still need to be determined.[22] Using the co-culture approach, Cooke et al[23] demonstrated neocartilage formation without hypertrophy and calcification. Co-culturing of chondrocytes and MSCs is a novel and promising approach in cartilage tissue engineering but requires further research and optimization. Although great advances in the field of cartilage tissue engineering have been made, an ideal protocol regarding cell source, scaffold, biochemical and physical factors has still not been established. One of the main challenges still remains unsolved: to produce a functional construct that mimics the complex structure and the zonal organization of cartilage.

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