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In vitro Evaluation of Biosynthetic Activity of the Human Osteoarthritic Chondrocytes Embedded in Collagen Gel

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Abstract

The aim of this study was to evaluate the capacity of human osteoarthritic chondrocytes to synthesize the cartilage matrix components: chondroitin sulfate (CS), keratan sulfate (KS) and colagens (COL). The study was performed on cells embedded in type II collagen gel. Specific COL staining and glycosaminoglycan (CS, KS) immunohistochemical examinations demonstrated that the osteoarthritic cartilage cells maintain their anabolic ability. Despite the biosynthetic activity displayed by chondrocytes, a net loss of the extracellular matrix represents the hallmark of all stages of osteoarthritic cartilage degeneration. This leads to the assumption that overall enzymatic degradation of the extracellular matrix components might be the reason for the metabolic imbalance in osteoarthritic cartilage.

Type II collagen gel obtained from bovine tracheal cartilage proved to be a good chondrocyte support for studying cartilage extracellular matrix turnover.

Keywords*:* osteoarthritis, chondrocyte, collagen gel, culture, chondroitin sulfate, keratan sulfate, collagen.

Introduction

Normal cartilage homeostasis of adults represents a fragile balance between extracellular matrix (ECM) components degradation and synthesis in order to maintain the joint functional integrity. Under the influence of mechanical stress or inflammatory articular process, this dynamic balance is disturbed and gradually the catabolic events become predominant leading to cartilage degradation and a progressive loss in joint function [1]. For a long time, osteoarthritis (OA) has been considered as a consequence of ageing or mechanical destruction of articular cartilage. However, this concept has been developed during the recent years with its discovery as a metabolically active tissue.

Biochemical changes occurring in OA cartilage affect its major ECM components: collagens and proteoglycans (PGs). Beside the degradation of these components, destabilization of supramolecular structure such as the collagen network and changes in the expression profile of matrix molecules also take place [3]. Thus, the composition of the proteoglycans has been shown to change in OA cartilage, such as an increase in the size of PG

molecules and the appearance of molecules, which are barely seen or are undetectable in normal articular cartilage. These atypical molecules are tenascin [4] and collagen types IIA [5] and III [6]. It still does not known if a loss of proteoglycans or a loosening of the collagen network comes first or both finally implicate the other as well [7].

Although precise mechanisms through which the joint cartilage develops characteristic OA lesions are not fully elucidated, it is increasingly accepted the involvement of mechanical and enzymatic factors based on studying of the OA animal models. These studies revealed matrix metalloproteinases (MMPs) as major enzymatic factors in this process [8]. Unlike inflammatory arthropathies, where infiltrated neutrophils and pannus invading the cartilage contribute to the degrading process through proteolytical enzymes able to resorb ECM, the OA lesion is characterized by reduced inflammation and synovial proliferation, orientating the investigations towards native chondrocytes as a source of MMPs. Thus, enhanced levels of numerous MMP members including MMP-7 [9], membrane type 1 (MT1)-MMP [10], aggrecanase [11], ADAM-10 [12] and ADAM-15 [13] have been shown to determine the increased ECM degradation in OA cartilage.

In this context, the main objective of the undertaken studies was the carrying out of an *in vitro* study model for biosynthetic activity of OA chondrocytes by: immunocytochemical revelation of chondroitin sulfate (CS) and keratan sulfate (KS) - the two glycosaminoglycans (GAGs) present in aggrecan structure and histological detection of the synthesized collagens in a three-dimensional system of collagen gel. This system creates the conditions for maintaining the phenotype expressed *in vivo* by chondrocytes [13].

Material and Methods

Biological material. Cartilaginous tissue samples were obtained under aseptic conditions from OA patients (mean age of 52 years) subjected to surgical intervention for hip joint prosthesis. According to macroscopically evaluation in Mankin systems [14], the OA overall degree at hip level was V, but the tissue samples were taken from regions with residual cartilage, representing III and IV degrees. The articular cartilage was isolated through dissection excluding synovial and bone tissues.

Chondrocyte culture in monolayer. For chondrocyte isolation from cartilaginous tissue, the cartilage fragments from OA cartilage were cut into small pieces of about 1-2 mm^3, washed 3-4 times in PBS and subjected to sequentially enzymatic treatment with hyaluronidase, trypsin and clostridiopeptidase A, seeded and multiplied into monolayer according to experimental protocol carried out by Goldring [15].

Chondrocytes were seeded at a cell density of 10^6 cells/ml in the growth medium (DMEM containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 0,5 μg/ml amphotericin B and incubated at 37^0 C in a humidified atmosphere of 5% CO_2. They required 2-3 days in order to adhere to support and to display. The culture medium was changed at 3 days period of time. The culture evolution was observed by phase contrast microscopy. After about a week, when the primary culture cells reached the confluence, they were individualized by 0.25% trypsin/1.0 mM EDTA treatment and reseeded at the same cell density. In the performed studies there were used chondrocytes in monolayer culture up to the third passage.

***Preparation of chondrocytes suspended in collagen gel*.** OA chondrocytes maintained in monolayer culture up to the third passage were trypsinized, centrifuged and embedded in a type II collagen gel obtained from bovine tracheal cartilage according to the procedure described by Negroiu et al. [16].

Type II collagen solution at a concentration of 4 mg/ml was dialyzed against distilled water, with several changes, at 4^0C for 48 hours and sterilized with UV light on ice for 4 hours. Freshly dissociated chondrocytes were suspended in collagen solution at a density of 10^6 cells/ml according to the Matsusaki et al. procedure [17] adapted by us. Final collagen concentration in the suspension thus obtained was of 3.2 mg/ml and the solution pH was 7.4.

1ml of collagen-chondrocyte suspension was distributed in the 35mm culture dishes and incubated at 37^0 C in a humidified atmosphere of 5% CO_2 for 1 hour, a necessary period to gelify collagen-cells suspension. The resulted gel was coated with 2 ml culture medium and it was incubated under the same conditions, for 10 days and the medium was changed every 2 days.

Evaluation of biosynthetic activity of the OA chondrocytes included in collagen gel. After 10 days of culture, the collagen-chondrocytes composite was washed with PBS and processed for further morphological evaluation by light and electron microscopy and CS and KS immunocytochemical detection, as it was previously mentioned [18].

Results and Discussion

The biochemical properties of articular cartilage are strongly dependent on the biochemical composition and integrity of its ECM. It is an avascular, aneural and alymphatic matrix, which is synthesized by the resident cells named chondrocytes [19]. At the supramolecular level, cartilage matrix consists of two basic components: a fibrillar and an extrafibrillar matrix. The fibrillar matrix consists predominantly of type II collagen, together with other collagens, mainly types IX, XI and VI [20]. The nonfibrillar component consists predominantly of highly sulfated aggrecan monomers, attached to hyaluronic acid and link protein, forming very large, polyanionic aggregates.

OA consists of the destruction and failure of the ECM, the functional element of articular cartilage. So far, the studies have been focused on changes in the two major components of the cartilage matrix, the collagen network and the proteoglycan aggregates. The loss of aggrecan is a feature in early stages of cartilage degeneration, whereas the overall content of collagen remains constant throughout the disease process [21].

Numerous studies performed on chondrocytes were focused on the understanding of the mechanisms inducing metabolic changes in joint cartilage during OA. Nevertheless, a major impediment in these studies is represented by the fact that cells isolated from their cartilaginous matrix environment and cultured in monolayer for 2-4 weeks are strongly inhibited through contact and gradually modify their characteristic phenotype, adopting an elongated morphology, typical for fibroblasts and being characterized by an intense proliferation. These morphological changes are accompanied by profound biochemical changes including a gradual modification from type II collagen synthesis to type I and III collagens and from synthesis of the PG specific to cartilage (aggrecan) to the PG typical for fibroblasts (versican) [22, 23]. Nevertheless, chondrocyte-specific phenotype can be re-expressed when these cells are embedded in three-dimensional solid support matrices such as agarose [24], collagen gels [25] or alginate microspheres [26]. This ability is limited nevertheless and it was suggested that the cells cultured in monolayer are not to be subjected to more than 4 passages in order to maintain their chondrocyte characteristics [27].

As it has already shown previously, OA chondrocytes are characterized by an intense degradative activity reflected by the synthesized proteolytic enzymes (particularly MMPs). Nevertheless, joint cartilage destruction is the result of the disturbance of the balance between degradation and synthesis of the matrix structural components. Therefore, the studies

undertaken by us had as purpose the evaluation of biosynthetic activity of the OA chondrocytes cultured in collagen gel that allows them to regain and maintain the phenotype expressed *in vivo*.

Numerous studies [28, 29] demonstrated that when chondrocytes were cultured in collagen gel in order to provide an three-dimensional structure ensuring the maintenance or the revert to their characteristic phenotype, they synthesize an ECM *de novo* represented by GAGs and type II collagen, thus constituting an architecture similar to hyaline cartilage. In order to reproduce as faithful as possible the existing *in vivo* condition, it was used this experimental model with the purpose to evaluate qualitatively the OA chondrocyte ability to synthesize these matrix components during 10 days-culture in the presence of a growth medium (DMEM containing 10% FCS).

In the performed studies, we used OA chondrocytes multiplied in monolayer culture up to the third passage. When the chondrocytes were isolated from OA cartilage and plated at high density (1x 10^6), typical polygonal chondrocytes were observed after 24 hours of culture and for the remaining 6 days of culture (data not shown). When the cells were propagated and cultured in monolayer they began to lose their polygonal shape and became more elongated and flattened. Thus, the OA chondrocytes at passage 3 were represented by a heterogeneous cell population (**Figure 1**) as a result of their partially dedifferentiation in monolayer culture and the phenotyping variability known to be expressed *in vivo* by OA chondrocytes [30].

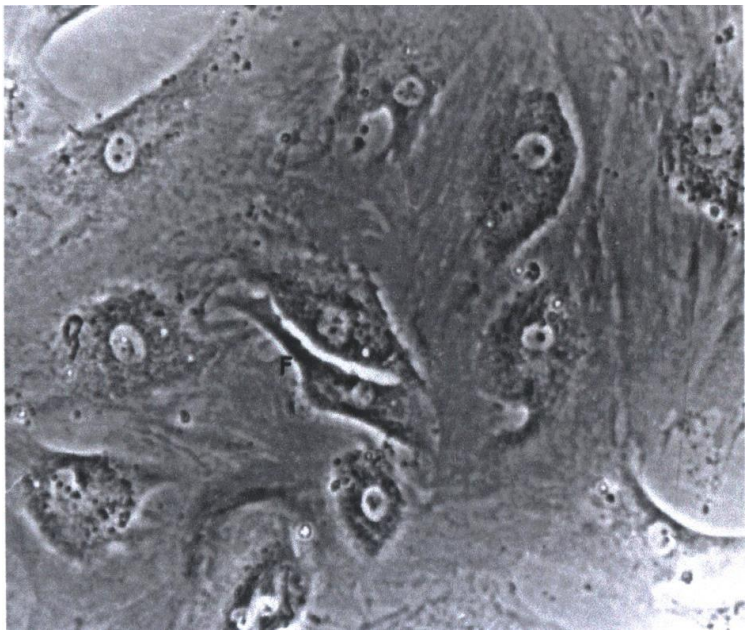

Figure 1. Appearance of human OA chondrocytes in culture (third passage), as viewed by phase contrast microscopy (x400). Cells partially lost their typical polygonal shape. F- fibroblast-like cell.

Histological studies revealed the fact that although most chondrocytes embedded in collagen gel exhibited their specific phenotype, extremely few cells underwent changes toward a fibroblast-like phenotype (**Figure 2**) adopting a slightly elongated morphology. This could be accounted for by the osteoarthritic nature of cartilage from which there were isolated the cells under study, some of them exhibiting an irreversibly altered phenotype. Similar to chondrocytes embedded in alginate microspheres, chondrocytes in collagen gel exhibited an extremely low proliferation speed compared to cells cultured in monolayer. Thus, rare cell colonies (**C**) were revealed.

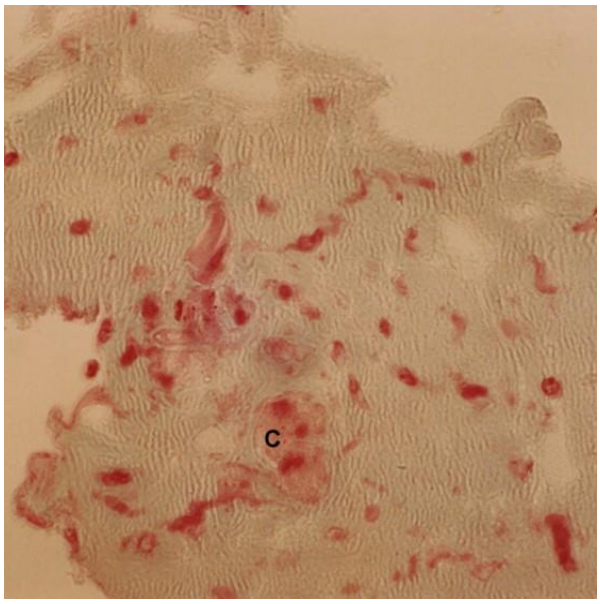

Figure 2. Light micrograph of OA chondrocytes-collagen gel composite.
OA chondrocytes embedded in collagen gel with a prevalently round morphology;
Cell cluster (C). (Azan staining).

Studies on immunohistochemical evaluation of chondrocyte-type II collagen composite demonstrated positive reactions both to CS (**Figure 3A**) and to KS (**Figure 3B**) prevalent at intracytoplasmic level. Reaction intensity varied depending on the cell, but was relatively homogeneous. The signal absence in pericellular microenvironment might suggest either low or late secretory activity of these cells, or an intense degradative activity.

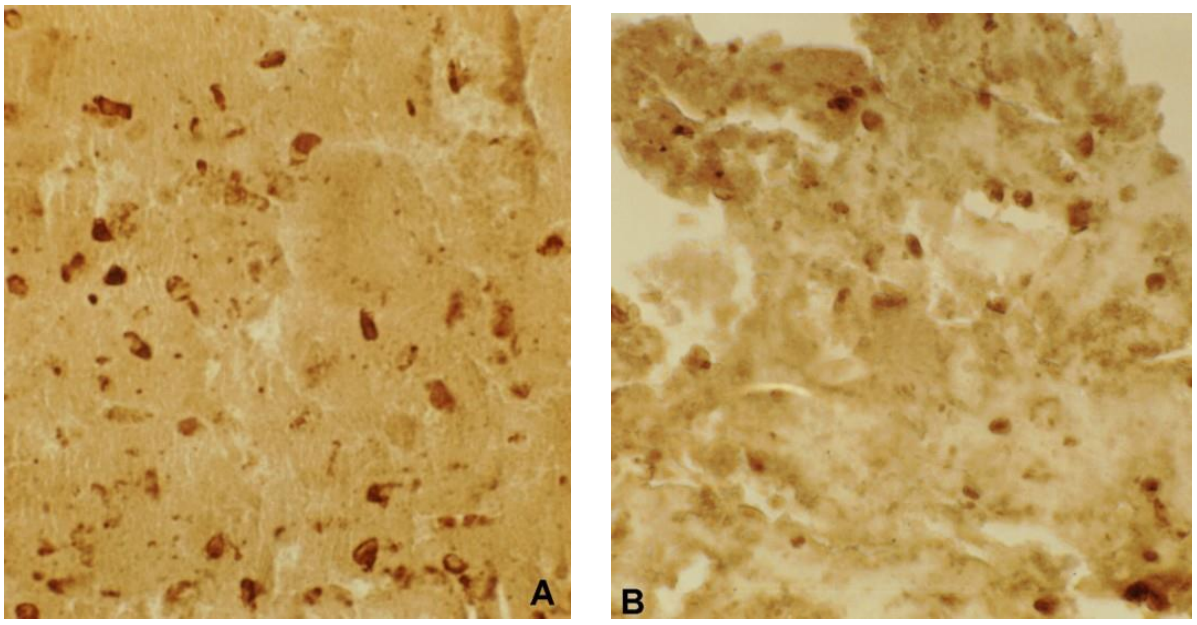

Figure 3. Immunohistochemical revealing of CS (A) and KS (B) synthesized by OA chondrocytes embedded in type II collagen gel.

Nevertheless, the van Gieson staining, specific to collagens was more intense in the proximity of some cells (COL) (**Figure 4**) suggesting their increased anabolic activity, which exceeds their degradative activity, thus permitting collagen pericellular depositing. Unfortunately, it is not known whether this was type I or type II collagen or possibly another collagen type.

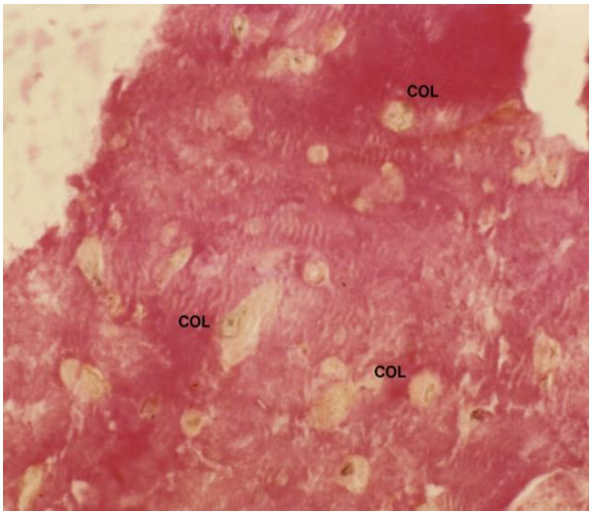

Figure 4. Light micrograph of an OA chondrocyte – type II collagen composite.
It can be noticed collagen deposit in the proximity of some chondrocytes (COL). (van Gieson staining).

Electron microscopy images (**Figure 5**) revealed chondrocytes of ellipsoidal shape with numerous membrane filopodia (f) specific to superficial region of joint cartilage. These cells are characterized by an abundant endoplasmic reticulum (er) to which ribosomes are attached and numerous secretory vesicles (v). This indicates active intracellular protein synthesis in these cells. There were also revealed round chondrocytes exhibiting a voluminous, euchromatic nucleus, with its chromatin condensed along its nuclear envelope (data not shown).

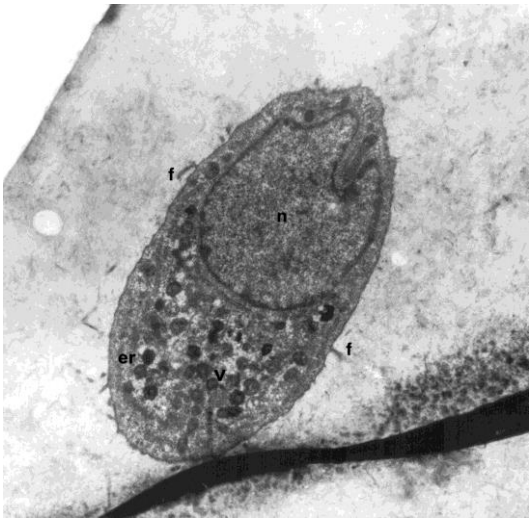

Figure 5. Electron micrograph of an OA chondrocyte embedded in collagen gel.
Ellipsoidal-shape chondrocyte with numerous mitochondria, secretory vesicles (v) and an abundant endoplasmic reticulum (er) to which ribosomes are attached. Nucleus (n). (x 15 000).

Conclusions

Type II collagen gel obtained from bovine tracheal cartilage proved to be an ideal culturing system for chondrocytes, since provides them with the possibility to regain and maintain their phenotype expressed *in vivo*. Thus, histological and electron microscopy studies revealed the fact that the third passage OA cartilage cells embedded in collagen gel, exhibited a morphology typical for chondrocytes.

Histological and immunohistochemical studies on synthesis of collagens and cartilage specific glycosaminoglycans (CS and KS) in solid, three-dimensional matrices of collagen gel demonstrated that OA cartilaginous cells are anabolically active. Consequently, net loss of ECM macromolecules during OA cartilage degenerative process is not due to their lack of synthesis, but to the increase in matrix catabolism.

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