University of Medicine and Pharmacy
Craiova

Ph D Thesis

IMPLICATIONS OF CHONDROGENESIS IN THE PREVENTION AND TREATMENT OSTEOARTHRITIS

ABSTRACT

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LIST OF ABBREVIATIONS

MSC – mesenchymal stem cells
PG – prostaglandins
IGF I – insulin-like growth factor I
ROA – radiographic osteoarthritis
DBM – demineralized bone matrix
TGF-β3 – transforming growth factor I
PCR – polymerase chain reaction
OR – odds ratio
CI – confidence interval
SD – standard deviation
INTRODUCTION

The most common disease affecting synovial joints is the arthrosic disease. It is a chronic disease that leads to the slow degeneration and erosion of the articular cartilage, causing pain and inability to use the affected joint.

Since data from the literature on the role of mesenchymal stem cells (MSC) in orthopaedics are insufficient, and taking in consideration their potential in studies in other fields, we considered appropriate to use them in the medical practice: osteoarthritis. Thus, our results could form the basis of the future more detailed studies that aim to decode the cellular mechanisms of interaction and differentiation of these cells.

The aim of this study is to contribute to the use of the mesenchymal stem cells in surgery, thus contributing to a more individualized treatment to each patient suffering from osteoarthritis. In this context, the main objectives of the thesis are:

1. to compare the protein and proteoglycan synthesis ability of normal and OA MSCs in vitro;
2. to evaluation the synthesis of proteoglycans (PG) in the arthrosic cartilage to the cartilage from the normal knee;
3. we studied the 192 bp allele of the IGF-I promoter polymorphism in relation to osteoarthritis, assuming that this polymorphism determines IGF-I expression both in blood and in cartilage; we also studied the interaction of
this genetic polymorphism with the COL2A1 polymorphism;

4. we tested the hypotheses that severity of osteoarthritis is correlated with aggrecan and collagen type II mRNA expression and that differences in expression can occur within an affected joint;

5. autologous chondrocyte transplantation analysis results in the treatment of cartilage injuries of the knee.

Keywords: osteoarthritis, mesenchymal stem cells, condrogenesis, collagen type II A1, IGF I, aggrecan, autologous chondrocyte transplantation.

I. REVIEW OF THE PRESENT KNOWLEDGE

Chapter 1 entitled "The structure and function of articular cartilage" analyzes the structure and formation of the synovial joints, the general structure of the articular cartilage and collagen components of extracellular matrix.
Chapter 2 - entitled "The use of mesenchymal stem cells in chondrogenesis" analyzes: the origin of tissue specific stem cells, mesenchymal stem cell biology and role, harvesting, isolation and culture of mesenchymal stem cells, mesenchymal stem cells and their applications in tissue engineering.

II. PERSONAL CONTRIBUTIONS

Chapter 3 - Chondrogenic potential of mesenchymal stem cells from patients with osteoarthritis.

The study was conducted in the Emergency Clinical Hospital Bucharest, Department of Orthopaedics and Traumatology, from January 2009 to March 2009 on 7 patients with osteoarthritis and 9 adults without any symptoms of osteoarthritis.

Mesenchymal stem cells were obtained by puncture of the postero-superior iliac spina, followed by isolation and cultivation of MSCs.

In vitro Cartilage Formation

For in vitro chondrogenesis studies, a novel micromass culture system was developed. Briefly, a total of $2 \times 10^5$ MSCs per well were placed in 200µl final volume in U-bottom tissue culture plates in DMED/F12 medium (GIBCO) supplemented
with 10% of a selected lot of FCS, $10^{-7}$ M dexamethasone, 10 ng/ml TGF-β3 and 6.25µg/ml insulin, with DBM alone or with DBM plus growth factors. The 96-well tissue culture plates were then centrifuged at 1,200 rpm and incubated at 37 °C in 5% CO₂. Within 24 h after incubation, the cells formed aggregates which did not adhere to the bottom of the plate. The medium was changed every 3–4 days, and cell aggregates were obtained at intervals of 14 days. Sulfated glycosaminoglycan was visualized with 1% 1,9-dimethyl-methylene blue.

For quantification of proteoglycan synthesis, cultures received 1.0µCi/well sodium $^{35}$S-sulfate and 1.0µ Ci/well sodium $^3$H-leucine 24 h prior to the time point chosen for measurement of newly synthesized proteoglycans and protein, respectively. Incorporation of radioactivity was measured by liquid scintillation counting.

Immediately after centrifugation, the cells appeared as flattened pellets at the bottom of the wells. One day later, the pellets became spherical without any increase in size. As the pellets grew in size between days 4 and 14, a cartilage matrix was synthesized, as demonstrated by the significant increase in the levels of $35$ S-sulfate incorporation at days 7 (fig. 1 a–c) and 14 (fig. 1 d–f) compared with untreated control cultures. Maximal level of sulfate incorporation occurred in cultures containing TGF-β3, insulin and DBM. Addition of TGF-β3 and insulin, or
DBM alone also resulted in a significant increase in the levels of sulfate incorporation, that is proteoglycan synthesis, compared to untreated samples. Furthermore, similar changes of type II collagen mRNA levels were demonstrated by quantitative RT-PCR analysis (fig. 1 g, h). After 14 days of chondrogenic culture, the extent of chondrogenesis was also assessed histologically using methylene blue (fig. 1 i) and toluidine blue (fig. 1 j) staining, as well as by transmission electron microscopy (fig. 1 k).
Fig. 1 – Chondrogenic differentiation of normal human MSCs. MSC aggregates were cultured either with medium alone, TGF-β3 plus insulin, DBM or TGF-β3 plus insulin in combination with DBM. $^3$H-leucine (a, d) and $^{35}$S-sulfate (b, e) incorporation was measured on days 7 (a–c) and 14 (d–f), then normalized sulfate incorporation was calculated (c, f). The expression levels of type II collagen and GAPDH were quantified by real-time RT-PCR on days 7 (g) and 14 (h). Results are expressed as means ± SD. i Methylene blue staining of a day 14 pellet maintained in the presence of TGF-β3, insulin and DBM. Scale bar = 1 mm. j Toluidine blue staining of a section from resin-embedded pellet cultured for 14 days in the presence of TGF-β3, insulin and DBM. Scale bar = 50µm. k Transmission electron micrograph depicting the ultrastructure of extracellular matrix inside the chondrogenic pellet on day 14. Scale bar = 50µm. Morphologically, 2 types of matrix could be observed.
Fig. 2 - Chondrogenic potential of normal and OA MSCs. MSC aggregates were cultured with TGF-β3 plus insulin in combination with DBM. $^3$H-leucine (a, d) and $^{35}$S-sulfate (b, e)
incorporation was measured on days 7 (a–c) and 14 (d–f), then normalized sulfate incorporation was calculated (c, f). The expression levels of type II collagen and GAPDH were quantified by real-time RT-PCR on days 7 (g) and 14 (h). Results are expressed as means ± SD. i Size and dry weight of representative pellets after 14 days of culture (h). Scale bars = 1 mm.

As can be inferred from figure 3, immediately after cartilage was obtained (day 0), the sulphate incorporation rate was higher for OA cartilage than for N cartilage. After culture, sulphate incorporation had increased for both N and OA cartilage. This increase was much larger for N cartilage than for OA cartilage.

![Graph showing sulphate incorporation rate of N and OA cartilage](image)

**Fig. 3 - Sulphate incorporation rate of N and OA cartilage immediately after the cartilage was obtained (day 0) and after...**
4 days of culture. Mean values + SEM are given, n = 4. A +B sum of the incorporation rate in the articular side (A) and the bone side (B). The white bar behind the hatched bar marked A+B represents the incorporation of an intact sample. *Differences with N cartilage are statistically significant. • Difference with the bone side is statistically significant.

Autoradiographic Localization of Sulphate Incorporation

Figure 4 shows the autoradiographic staining in the superficial, middle, and deep zone of N and OA cartilage samples, immediately after the cartilage was obtained (at day 0) and after 4 days of culture. The autoradiographs (Figure 5a-d) illustrate the findings in Figure 4. Immediately after cartilage was obtained, sulphate incorporation was mainly located in the middle and deep zone, not significantly different for N and OA cartilage (Figure 4, 4a, 4b). After culture for N cartilage, a significant relative increase in the sulphate incorporation occurred in the superficial zone. This resulted in a sulphate incorporation that was equally spread over the entire sample (Figure 4). This change in localization of sulphate incorporation in N cartilage samples after culture is also clearly illustrated by comparing Figure 5c and 5a. For OA cartilage, however, no significant change in the localization of sulphate incorporation after culture was observed. Sulphate incorporation was still located in the middle and deep zone of the cartilage (Figure 4).
Fig. 4. Autoradiographically determined relative $^{35}$S-sulphate incorporation in the superficial (S) middle (M) and deep (D) zone.

In the present study (Jalbă BA și colab., 2009), we have used a new micromass culture system, carried out in 96-well tissue culture plates, that facilitates the chondrogenic differentiation of human MSCs. We have found that culture-expanded MSCs isolated from OA patients do not differ significantly from the normal population in respect to their chondrogenic differentiation in vitro as far as the total protein and
proteoglycan synthesis as well as the expression of type II collagen mRNA are concerned.

**Fig. 5. Autoradiographs after 35S-sulphate incorporation; (a) N, day 0; (b) OA, day 0; (C) N, day 4; (d) AO, day 4 (magnification x50). Thue superficial (S) middle (M) and deep (D) zone are indicated. The inset in (d) shows a magnification (X250) of the cell cluster.**
We compared the PG synthetic activity of mild-to-moderate OA cartilage to healthy N cartilage of the human knee. The OA cartilage samples as used in this study can histologically be divided into two segments. The articular side (superficial layer) close to the joint space is damaged and has a severe reduction of PG content, and most chondrocytes are joined in cell clusters. The side near to the bone (middle and deep zone) seems histologically intact and similar to N cartilage samples. In this segment, cell clusters were not observed.

Immediately after the cartilage is obtained, samples of OA cartilage have a higher PG synthesis than N cartilage.

**Chapter 4** is entitled „The interaction between the IGF I gene and collagen type II A1 in radiographic osteoarthritis“.

We studied the 192 bp allele of the IGF-I promoter polymorphism in relation to osteoarthritis, assuming that this polymorphism determines IGF-I expression both in blood and in cartilage; we also studied the interaction of this genetic polymorphism with the COL2A1 polymorphism (Bijkerk C, 1999).

In this study population 14 out of 23 reported alleles of the COL2A1 VNTR polymorphism were detected. In table 1, allele frequencies in all genotyped subjects, as well as the allele frequencies in ROA cases and referent subjects, both overall and stratified by gender and site of ROA, are shown. In the overall
analysis, the frequency distribution of alleles in ROA cases and referent subjects was different, although this difference was borderline significant (p ~ 0.06). In women the allele distribution in ROA cases as compared to referent subjects was statistically significantly different (p ~ 0.03). The difference in allele distribution between female ROA cases and referents was explained by an increased frequency of allele 13R1, while the frequencies of all other alleles, except allele 14R2, were found to be decreased or similar in cases and referent subjects. In men no significant differences were found between ROA cases and referent subjects, although the allele frequency of allele 13R1 in male cases was slightly elevated (except for male cases with knee ROA).

**Table 1. Allele frequencies of the COL2A1 VNTR polymorphism**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>13R1+/13R1−</th>
<th>13R1+/13R1+</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OR (95% CI)</strong></td>
<td><strong>OR (95% CI)</strong>†</td>
<td><strong>OR (95% CI)</strong>*</td>
</tr>
<tr>
<td><strong>Cazurile cu ROA și noduli Heberden</strong></td>
<td>1,53 (0,91-2,60)</td>
<td>1,69 (0,97-2,94)</td>
</tr>
<tr>
<td><strong>Barbati</strong></td>
<td>1,31 (0,53-3,26)</td>
<td>1,16 (0,45-2,96)</td>
</tr>
<tr>
<td><strong>Femei</strong></td>
<td>1,64 (0,87-3,12)</td>
<td>1,97 (1,00-3,90)</td>
</tr>
<tr>
<td><strong>GOA</strong></td>
<td>1,62 (0,73-3,59)</td>
<td>1,63 (0,67-3,46)</td>
</tr>
<tr>
<td><strong>Barbati</strong></td>
<td>1,60 (0,29-8,81)</td>
<td>1,41 (0,23-8,71)</td>
</tr>
<tr>
<td><strong>Femei</strong></td>
<td>1,60 (0,65-3,93)</td>
<td>1,59 (0,62-4,09)</td>
</tr>
</tbody>
</table>
In this population based study we found that the absence of the 192 bp allele of a microsatellite polymorphism in the promoter region of the IGF-I gene was associated with increased prevalence of radiographic osteoarthritis in subjects aged 65 years or younger. Compared with homozygotes for the 192 bp allele, the prevalence of ROA was higher in heterozygotes (OR 1.4 (95% CI, 1.0 to 1.8)) and non-carriers of the allele (OR 1.9 (1.1 to 3.3)). This effect most probably occurs in interaction with the COL2A1 gene, as the prevalence of ROA increased in individuals with the risk genotype of both genes.

Chapter 5 – Alteration of cartilage specific genes, aggrecan și colagen II, în osteoarthritis.

This study tests the hypothesis that disease severity is characterized by alterations in expression of cartilage-specific genes for aggrecan and collagen type II.

Cartilage, discarded from six subjects undergoing knee replacement, was subdivided into homogeneous portions by the surgeon according to the Outerbridge classification. For four subjects, it was possible to separate the tissue into two or three fractions with different disease severity. Portions of each sample were prepared either for histological analysis and ranking according to the Mankin system or for RNA extraction. Quantitative, competitive RT-PCR assays were used for
measurement of mRNA for aggrecan, collagen type II, and glyceraldehyde-3-phosphate dehydrogenase. Clinical grading was correlated with histological score. There was a striking decrease in expression of aggrecan and collagen II that was correlated with increase in the grade in regions of cartilage within an individual subject. In the series of 12 samples, there was an inverse correlation between aggrecan expression and osteoarthritis grade.

**Fig. 6 - Correlation between histological Mankin score and osteoarthritis (Outerbridge) clinical grade (OA).**

$r = 0.60, p = 0.043$
Fig. 7 - Correlation between aggrecan gene expression and osteoarthritis (OA) clinical grade for all 12 samples.

Capitolul 6 is entitled “Autologous chondrocyte transplantation in the treatment of cartilage injuries of the knee”.

Once a patient is determined to be eligible for this procedure, during an arthroscopic examination, his orthopedic surgeon takes a biopsy of the patient's articular cartilage. This sample, between 200 mg and 300 mg, is removed from an area of the knee that is not weight-bearing, usually the femoral condyle. Chondrocytes are separated from their surrounding cartilage and cultured for four to five weeks, generating between 5 and 10 million cells. The procedure to implant the cells is a surgical procedure in which the patient's joint is exposed by the orthopedic
surgeon. The defect area is prepared by removing dead cartilage and smoothing the surrounding living cartilage (fig. 8).

Fig. 8. Image of the defect area after removing dead cartilage.

A piece of periosteum, the membrane which covers bone, is taken from the patient's tibia and sutured over the prepared defect (figure 9). The cultured chondrocytes are injected by the surgeon under the periosteum, where they will grow and mature over time.

One patient who had a preoperative Lysholm knee score of 46 and a chondral defect of 4cm² following 2 previous debridements elected to have a total knee replacement 5 months postoperative. Mean Lysholm score of the remaining 15 patients increased from 43 before treatment to 85 at 1 year postsurgery. 18 of these (90%) had an improved knee score. 7 of the 9 patients who have been followed up for 2 years retained this improvement
or had a further increase in knee score. Two patients who have reached 3 years post–surgery had knee scores of 95 and 100 respectively. 82% patients were pleased or extremely pleased with the outcome at most recent follow-up and several have returned to competitive sport. No statistically significant correlation between the patients’ age and the success of the ACT procedure was found.

Fig. 9. Transfer of the periosteum over the prepared defect.

Chapter 7 – General conclusions

1. our results indicate that autologous MSCs from BM of RA and OA patients might be used for cartilage and/or bone replacement therapy in the future.
2. The absence of the 192 bp allele in the promoter region of the IGF-I gene is associated with increased prevalence of radiographic osteoarthritis before the age of 65 years.

3. The study also suggests the possibility of a genetic interaction between the IGF-I and the COL2A1 genes in the occurrence of this disease.

4. This study shows a significant relationship between expression of aggregcan and regional disease severity in osteoarthritis. Within a subject, there were relationships between disease severity and relative mRNA expression for aggregcan as well as for collagen type II. For the whole series of samples, the inverse relationship between aggregcan expression and disease severity prevailed.

5. Finally, we show that use of quantitative, competitive RT-PCR is a useful research tool to assess chondrocyte gene signatures in small amounts of human cartilage.
SELECTIVE BIBLIOGRAPHY

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Foreign languages: English, German

Papers published

I. Papers published ISI:
1. Adela Laura Popa, Anca-Daniela Vlădoi, Mihoc Fl., and Jalbă
   B.A. Measuring Patient Satisfaction Within Romanian
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II. Papers published CNCSIS:


III. Scientific papers presented


