Identification of phenotypic discriminating markers for intervertebral disc cells and articular chondrocytes

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Objective. The present study was conducted to improve our knowledge of intervertebral disc (IVD) cell biology by comparing the phenotype of nucleus pulposus (NP) and annulus fibrosus (AF) cells with that of articular chondrocytes (ACs).

Methods. Rabbit cells from NP and AF were isolated and their phenotype was compared with that of AC by real-time PCR analysis of type I (COL1A1), II (COL2A1) and V (COL5A1) collagens, aggrecan transcript (AGC1), matrix Gla protein (MGP) and Htra serine peptidase 1 (Htra1).

Results. Transcript analysis indicated that despite certain similarities, IVD cells exhibit distinct COL2A1/COL1A1 and COL2A1/AGC1 ratios (Htra1).

Conclusions. Our study shows that NP and AF cells exhibit a clearly distinguishable phenotype from that of AC. Type V collagen, MGP and Htra1 could greatly help to discriminate among NP, AF and AC cells.

KEY WORDS: Intervertebral disc, Nucleus pulposus, Annulus fibrosus, Chondrocyte, Phenotype.

Introduction

Although intervertebral discs (IVDs) are thought to contribute to low-back pain, the cellular and molecular mechanisms that govern their formation, growth, maturation and ageing of IVD are still somewhat poorly documented. IVDs are complex anatomical structures with specific load-bearing organization that provide the spine with flexibility. IVDs are composed of three morphologically distinct regions: the annulus fibrosus (AF) surrounding a central nucleus pulposus (NP) and the inferior and superior cartilage endplates. Cells of the AF are generally considered as fibroblast-like, given their elongated and thin shape as well as the synthesis of type I collagen [1–6]. The central NP is a highly hydrated gel-like matrix composed of randomly organized proteoglycans and radially oriented collagen and elastin fibres [1–4]. Given their spherical shape and ability to express type II collagen and aggrecan, the two major markers of chondrocytic cells, cells of the NP are commonly characterized as chondrocyte-like cells [5, 6]. As regards to these characteristics, IVD is traditionally considered as a fibro-cartilaginous tissue with a composition relatively similar to that of articular cartilage. However, considering the embryological origin and the cell biology of IVD, this traditional characterization appears to be an oversimplification and is still a matter of much debate [7]. From the tissue-level perspective, the structure of the proteoglycans differs in both perspectives, the structure of the proteoglycans differs in both. As regards to these characteristics, IVD is traditionally considered as a fibro-cartilaginous tissue with a composition relatively similar to that of articular cartilage. However, considering the embryological origin and the cell biology of IVD, this traditional characterization appears to be an oversimplification and is still a matter of much debate [7]. From the tissue-level perspective, the structure of the proteoglycans differs in both tissues. Functionally, such differences help to endow IVD and articular cartilage with certain distinct biomechanical properties [8]. In light of these apparent discrepancies at both the histological and biomechanical levels, it seems reasonable to ascertain whether articular cartilage and IVD share any biological features. The present study was conducted to address this issue and to provide new insights into the biology of IVD cells. To this end, the phenotype of IVD cells and articular chondrocytes (ACs) was compared by real-time PCR. We also endeavoured to find specific markers to discriminate each cell type in order to establish a phenotypic signature for IVD cells.

Materials and methods

Materials

Hyaluronidase, trypsin, type II collagenase and isopropanol were purchased from Sigma-Aldrich (St Louis, MO, USA). Hanks balanced sodium salt (HBSS), DMEM (4.5 g/l glucose), Trizol and Dnase I were obtained from Invitrogen (Paisley, UK). Avian myeloblastosis virus-reverse transcriptase (AMV-RT), random hexamers and recombinant ribonuclease inhibitor were provided by Promega Corporation (Madison, WI, USA). Brilliant SYBR Green Master Mix was obtained from Stratagene (La Jolla, CA, USA). PCR primers were synthesized by MWG Biotech (Ebersberg, Germany).

Isolation of articular and IVD cells

Rabbit ACs were isolated from the femoral and humeral head cartilage as described previously [9]. For AF and NP cell isolation, AF and NP tissues were separated and enzymatically digested by 0.05% hyaluronidase in HBSS at 37°C for 10 min, then with 0.2% trypsin for 15 min and with 0.2% type II collagenase for 30 min [7]. The different resuspended rabbit cells were frozen for subsequent real-time PCR analysis.

Reverse transcription and real-time PCR

As previously described [10], total RNA was extracted using Trizol reagent according to the manufacturer’s instructions.
After deoxyribonuclease I digestion, RNA samples (2.5 μg) were reverse-transcribed using AMV-RT and random primers in a total volume of 30 μl. cDNA was amplified in a total volume of 25 μl PCR reaction containing 12.5 μl of brilliant SYBR Green Master Mix (1x) and 30 nM of SYBR green reference dye. The primer sequences used for RT-PCR are as follows: β-actin forward primer: 5’-ccctctacaggtggtcg-3’; β-actin reverse primer: 5’-ctctgtgtgctccgcaag-3’; type I collagen (COL1A1) forward primer: 5’-aggtatgctgcatccag-3’; COL1A1 reverse primer: 5’-tgcatgtgctggtatc-3’; type II collagen (COL2A1) forward primer: 5’-acagcggtttacccactcag-3’; COL2A1 reverse primer: 5’-ccaccatcaggggggtcc-3’; type V collagen (COL5A1) forward primer: 5’-gtcttgatgtcccgcacg-3’; COL5A1 reverse primer: 5’-cccaagttgtagtggctg-3’; aggrecan transcript (AGC1) forward primer: 5’-gaggtatgctgctggtatc-3’; AGC1 reverse primer: 5’-tgggtctctctctctc-3’; matrix Gla protein (MGP) forward primer: 5’-cloninatagctctatc-3’; MGP reverse primer: 5’-tttaccttacttacttact-3’; HtrA serine peptidase 1 (HtrA1) forward primer: 5’-ggcttcagaggaggcttc-3’; and HtrA1 reverse primer: 5’-gacttaccttactggtggag-3’. Real-time PCR was performed in the Mx3000P QPCR System (Stratagene) as previously described [10]. Cycle thresholds were normalized to β-actin in order to verify cDNA quantification differences. Results are reported as fold change in gene expression relative to control conditions.

**Statistical analysis**

Each experiment was repeated three times. Results are expressed as mean ± S.E.M. of triplicate determinations. Comparative studies of means were performed by using one-way analysis of variance followed by a post hoc test (Fisher’s projected least significant difference) with a statistical significance at P < 0.05.

**Results**

To address the controversy surrounding the phenotype of AF and NP cells, we sought to compare the expression levels of the major chondrocytic markers in freshly isolated AC and IVD cells (Fig. 1). AGC1 mRNA expression was slightly higher in NP and AF cells than in AC, with a 1.4- and 1.6-fold higher expression (Fig. 1A). In contrast, COL2A1 mRNA expression was significantly lower in AF and NP cells than in the AC, with a respective 2- and 34-fold decrease (Fig. 1B). The expression of COL1A1 mRNA was 17-fold lower in NP cells than in AC, whereas AF cells demonstrated the highest expression level that was more than 37-fold higher than AC. These results show that AF cells expressed the highest level of AGC1 and COL1A1 mRNA and that AC expressed the highest level of COL2A1 mRNA, whereas NP cells expressed a low level of transcripts coding for COL1A1 and COL2A1 and a high level of AGC1 mRNA.

To understand the comparison between the phenotype of IVD cells and AC better, we also expressed our data as a ratio of COL2A1/COL1A1 and COL2A1/AGC1 mRNA expression (Fig. 1D and E). The COL2A1/COL1A1 ratio was high for AC (1790) and for NP cells (930), indicating that COL2A1 mRNA expression largely predominates over that of COL1A1. On the contrary, AF cells exhibited a COL2A1/COL1A1 ratio of approximately 26, indicating that the expression of COL2A1 in AF cells was also predominant but to a significantly lesser extent. The ratio of COL2A1/AGC1 mRNA expression was about 1090 for AC and 370 for AF cells, indicating the predominance of COL2A1 mRNA expression over that of AGC1. On the contrary, NP cells exhibited a very low COL2A1/AGC1 ratio (23), showing that AGC1 was significantly higher in NP cells as compared with AF cells and AC.

To improve the discrimination and the characterization of the three cells types, we sought to identify several complementary markers that may have a differential expression pattern in AC and IVD cells. The expression of transcripts coding for COL5A1, the MGP and HtrA1 was thus evaluated and compared by real-time PCR.

Our results show that COL5A1 was predominantly expressed in AF cells, whereas it was barely detectable in AC and NP cells (Fig. 2A). As expected, we found a high expression level of MGP in AC. Surprisingly, AF cells showed only slightly detectable expression levels of MGP, whereas NP cells expressed undetectable levels of MGP transcripts (Fig. 2B). Finally, IVD cells exhibited a high level of HtrA1 mRNA expression as compared with AC (Fig. 2C). These results suggest that COL5A1 may be a potential marker for AF cells, whereas NP cells could be characterized by a high expression of HtrA1 and a lack of MGP expression.

![Fig. 1. Phenotypic characterization of ACs, AF cells and NP cells. Total RNA was purified from freshly isolated AC, NP and AF cells. Real-time PCR analysis for aggrecan (AGC1) (A), type II collagen (COL2A1) (B) and type I collagen (COL1A1) (C) was performed as described in the 'Materials and methods' section. Results are reported as fold change in gene expression relative to AC. For (D) and (E), results are reported as ratio of type II collagen to type I collagen mRNA expression (COL2A1/COL1A1) and ratio of type II collagen to aggrecan mRNA expression (COL2A1/AGC1). *P < 0.05 compared with AC.]
discussion

Increasing attention has been paid to the regeneration of functional tissue by cell therapy and/or tissue engineering, particularly for the NP, the primary structure affected in the degenerative IVD process [11]. As a prerequisite to the development of tissue engineering strategies, it appears crucial to generate basic knowledge regarding the biology and physiopathology of the targeted tissues. Particularly noticeable with IVD, the lack of molecular and cellular data mean that IVD tissue engineering is a huge challenge. To address this issue, the objectives of this study were to obtain new insights into the phenotypic characterization of AF and NP cells. An over-simplification [5, 6, 12, 13] has, however, attributed a phenotype to AF and NP cells which is close to that of fibroblasts and chondrocytes, respectively. On the one hand, AF and NP cells do not share a common embryological origin, making it difficult to assume a common phenotype. Cells from the AF and AC are derived from the mesoderm, whereas cells of the NP originate from the notochord [14]. On the other hand, the histological organization of IVD and its biomechanical properties are quite different from those of articular cartilage. It therefore seems reasonable to speculate that tissular adaptation to the mechanical constraints in IVD and articular cartilage leads to a different qualitative composition and cell phenotype.

To investigate the phenotype of rabbit IVD cells, the expression of the main chondrocytic markers was compared between freshly isolated AC and IVD cells. The supposedly chondrocyte-like phenotype of the NP cells was confirmed by the expression of type II collagen and aggrecan mRNA, whereas AC cells exhibited a fibroblastic phenotype indicated by the predominant expression of type I collagen. Nevertheless, type II, type I collagen and type II collagen/aggrecan ratios for AC, NP and AF cells were quite different, thereby indicating that NP cells exhibit an aggrecan-like phenotype in contrast to AC which exhibit a collagen-like one. Our histological analysis (data not shown) confirms that rabbit AF is a lamellar collagen-rich tissue containing GAG, whereas NP is a GAG-rich tissue containing type II collagen. Both tissues exhibit a histological organization quite similar to that of human IVD [15, 16].

In order to pursue the clarification of IVD cell phenotype, we were also interested in finding phenotypic markers able to discriminate the three cell types. As a result, the expression of type V collagen, a fibrillar collagen, was evaluated. The presence of this collagen has been previously suggested in articular cartilage and IVD [4, 12, 15, 17], but without discrimination between AF and NP cells. Here we found that the expression of type V collagen was drastically higher in AF cells compared with the other cells. Consequently, these data highlight type V collagen as a potential marker for AC cells that could, in addition to type I collagen, help improve the characterization of these cells.

Articular cartilage and IVD are both non-calcified tissues in which the mineralization process is likely to be tightly controlled. In articular cartilage, two inhibitors of calcification are involved in the control of mineralization: MGP and HtrA1 [18, 19]. Given the similarities between articular cartilage and IVD, we compared the expression of MGP and HtrA1 in both these tissues. As expected, our results showed an intense expression of MGP in ACs, whereas surprisingly MGP expression was barely detectable in AF cells and absent in NP cells. These results confirm those of Lee et al. [20] obtained in rats of a similar age. Contrary to the articular cartilage, the absence of calcification in the NP probably cannot be attributed to MGP. Interestingly, NP and AF cells showed a higher level of HtrA1 expression as compared with AC. This result led us to hypothesize that, although MGP is probably an inhibitor of calcification in articular cartilage, HtrA1 could be a potential inhibitor of IVD mineralization. Additional experiments must be performed to conclude on the role of HtrA1 in the control of IVD calcification, such as in age-dependent degenerative processes. Substantial age-related changes could be investigated in our rabbit model and compared with those obtained in rats by Lee et al. [20]. Lee et al. reported an increased expression of MGP in NP cells of aged rats. The potential effect of age on HtrA1 expression has not yet been described but should be evaluated.

In addition, given the similarities between the physiopathology of OA and disc degeneration and the putative role for MGP and HtrA1 in OA [19], the evolution of MGP and HtrA1 expression during age-dependent IVD degeneration deserves further consideration [19, 21].

With respect to the identification of discriminating markers for IVD cells and ACs, it remains to be clarified whether type V collagen, MGP and HtrA1, together with CD24 [22], HIF-1α [23–25], GLUT-1, MMP-2 [25], annexin A3, glycopic 3, keratin 19, pleiotrophin [20] and versican [26] could help piece together the puzzle of NP cell phenotype.

In conclusion, our study provides new insight into the IVD cell phenotype and shows certain similarities between AC and IVD cells. However, on the basis of type I, II and V collagen as well as aggrecan, MGP and HtrA1, we were able to propose a phenotypic signature for IVD cells. This phenotypic signature could become instrumental in monitoring and potentially triggering mesenchymal stem cell differentiation towards IVD cells.

rheumatology key messages

- NP cells are chondrocyte-like cells.
- MGP, Htra1 and type V collagen are discriminating markers for IVD cells.
- The phenotypic signature of IVD cells is instrumental for tissue engineering.

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