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Metabolic labelling of primary culture human cartilage cells to analyze the effect of Interleukin-1β in the extracellular matrix metabolism

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Introduction

Interleukin-1β (IL-1β) is a proinflammatory cytokine that acts as an arthritic mediator with the capacity to drive the key pathways typically associated with osteoarthritis (OA) pathogenesis. This degenerative joint disease, characterized by articular cartilage degradation, involve in its initial stages an increased cellular proliferation and synthesis of matrix proteins, proteinases, growth factors, cytokines and other inflammatory mediators by cartilage cells. Therefore, research has focused on the chondrocyte (the unique cell type of cartilage) as the cellular mediator of OA pathogenesis. Chondrocytes of OA patients, as well as synovial cells, produce increased levels of inflammatory cytokines, such as IL-1β [1]. This cytokine shifts the biology of the chondrocytes towards articular cartilage degradation by increasing the expression of matrix metalloproteinases while inhibiting the expression of genes encoding essential components of the cartilage extra cellular matrix (ECM).

We have recently used 2-DE technology to describe the intracellular proteome of normal and OA human chondrocytes [2-3]. In the present work, we have standardized the stable isotope labelling by amino acids in cell culture (SILAC) technique on primary culture human articular chondrocytes, and have applied for the first time this strategy for studying the chondrocyte extracellular matrix metabolism in basal conditions and under the effect of the proinflammatory cytokine IL-1β.

Methods

Cartilage obtained from patients undergoing joint replacement was provided by the Tissue Bank at

CHU A Coruña. The study was approved by the local Ethics Committee. Chondrocytes released from cartilage by enzymatic digestion were recovered and plated at low density in SILAC™ DMEM-Flex (Invitrogen) supplemented with antibiotics, glucose and 10% FBS. In the case of light media, standard L-lysine (146 mg/L) and L-arginine (28 mg/L) were used, while in the heavy media isotope-labelled L-lysine ($^{13}C_6$), and isotope-labelled L-arginine ($^{13}C_6$,$^{15}N_4$) were used. Titration assays were performed to minimize the problematic conversion of heavy Arg to Pro in chondrocyte cell culture. Cell proliferation and cell viability was tested by cell count and Trypan Blue dye exclusion. Real-time PCR analyses were carried out to verify the expression of Collagen II, a typical marker of chondrocyte cells, under the conditions of study.

Chondrocytes were used at week 2-3 in primary culture (P1), after making them quiescent by incubation in a medium containing 0% FBS for 24h. Cells were then washed thoroughly and incubated in serum-free medium with IL-1β at 5 ng/mL for 48 hours. Then, conditioned media were collected and their proteins were concentrated and quantified (Figure 1).

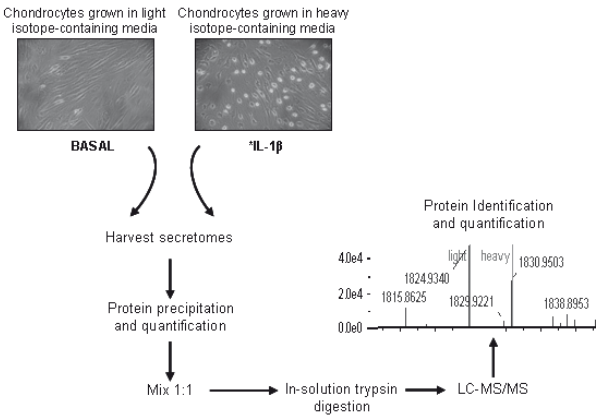

Figure 1. *Experimental workflow for the differential secretome analysis of chondrocytes by SILAC and nanoLC-MS/MS.*

Heavy and light samples were mixed 1:1, and 4 µg of each mixed sample were in-solution reduced, alkylated and digested with trypsin. Peptide mixtures were desalted and filtered through a C18 microcolumn, and column eluates were subjected to nano-LC-MS analysis using a Tempo nanoLC equipped with a C18 column, and a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems). The identification and quantification of proteins was carried out with Protein Pilot software.

Results

Chondrocytes are responsible of the synthesis of cartilage ECM components, but in primary culture tend easily to de-differentiate into fibroblasts. A method for obtaining a complete labelling of the proteins avoiding the cellular de-differentiation has been developed. We have minimized the Arg concentration in order to render it metabolically unfavourable as a precursor for Pro synthesis, and verified that these restricted conditions do not compromise cell proliferation, viability and expression of chondrocyte markers such as Collagen II, which is a proline-rich protein.

Collection and proteomic analysis of the proteins secreted by the chondrocytes allowed the identification of a number of proteins that had been previously related with OA pathogenesis, such as matrix metalloproteinases and cathepsins. Interestingly, most of the proteins identified are cartilage ECM components (27%) (Figure 2), clearly showing the usefulness of secretome analysis for the study of ECM metabolism. We could also find a high number of growth factors, matrix remodelling proteins and cytokines (6 of them that are known to be involved in the inflammatory response) that are secreted by IL-1β-treated chondrocytes, indicated those cellular pathways that are induced by this proinflammatory cytokine in cartilage cells.

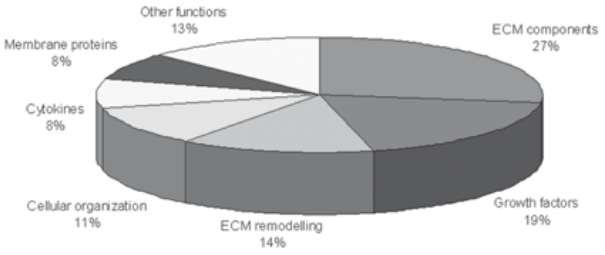

Figure 2. *Functional classification of the proteins identified in this work.*

Conclusions

We have standardized the SILAC technique for the proteomic analysis of human primary cartilage cells, and we have applied this technology for studying the effect of the proinflammatory cytokine IL-1β, a key molecule in the OA process, on the chondrocyte secretome. The obtained information will increase knowledge about OA pathogenesis, and already points out a number of possible markers of the disease.

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Identification of differential proteins in liver cells upon depletion of prohibitin

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Introduction

Prohibitin (Phb) is a multifunctional protein participating in a plethora of essential cellular functions, such as cell signalling, apoptosis, survival and proliferation, playing a central role in the maintenance of liver homeostasis through the regulation of central proteins involved in these processes by means of protein-protein interaction mechanisms. In the liver, deficient prohibitin activity participates in the progression of non-alcoholic steatohepatitis (NASH) and obesity, according to mechanisms that still must be elucidated. Phb1 plays an essential role in hepatic functions such as inhibition of cell proliferation [1, 2] and response to anti-cancer [3] or carcinogenic agents [4]. Down-regulation of Phb1 is an early event in the development of NASH and hepatocellular carcinoma (HCC) in experimental mouse models and humans [5]. All these data suggest that Phb1 may play a prominent role in the progression of HCC.

Materials and Methods

Phb1 expression was silenced in the human HCC cell line PLC/PRF5 using specific siRNAs (siGL, control cells; siPHB, treated cells). Phb1 levels were analysed by immunodetection assay. Apoptosis was quantified with the Cell Death Detection Kit (Roche). Cell proliferation was estimated by cell counting and with the Cell Proliferation Reagent WST-1 (Roche). In order to explore the molecular mechanisms involved in the response of PLC cells to impaired Phb1 activity we used a combination of DIGE and mass spectrometry analyses. To increase the efficiency of the process, cytosolic and microsomal subcellular fractions were isolated (Qproteome Cell Compartment Kit, from Qiagen). Enriched cytosolic and microsomal fractions from siGL and siPHB cells were compared by DIGE analysis. Differential spots detected and quantified by DeCyder were identified by nanoLC-ESI-MS/MS (Q-TOF).

Results

Phb1 levels were reduced by 80% upon siRNA silencing. Apoptosis was quantified and absorbance was 3-fold increased in siPHB cells indicating the proapoptotic effect of Phb deficiency (Figure 1D). In addition, Phb silencing severely compromised the capacity of PLC cells to proliferate in a semisolid substrate, an inherent property of transformed cells that is related with their metastatic capacity (Figure 1C). Both cell counting and formazan production revealed a significant reduction of the proliferation rate of siPHB cells when compared with the control siGL cells (Figure 1A and B).

DeCyder analysis of DIGE of cytosolic and microsomal fractions found 76 and 25 spots diffe-