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Effect of hyaluronic acid microparticles on primary human articular chondrocyte phenotype

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ABSTRACT

Cartilage is a specialized connective tissue performing many essential functions in the musculoskeletal system. If left untreated, cartilage injuries can lead to early progression of degenerative osteoarthritis (OA). Ideally, the efficient treatment of OA will require not only therapeutics that will reduce the degenerative processes, but also that will promote regeneration of cartilage. We produced hyaluronic acid (HA) microparticles to be appllied as a carrier for the delivery of therapeutic or signaling molecules (e.g. soluble growth factors) that can regulate cell function and cartilage regeneration. Viability and proliferation values of human articular chondrocytes in presence of HA microparticles (direct an non-direct contact) did not present significative changes compared to those in total absence of HA microparticles. In presence of HA microparticles, we observed an increase expression of aggrecan, SOX9 and COMP over time, which was not verified in the absence of HA microparticles. No significant changes on the expression of CD surface antibodies markers (CD 44, 90 and 105) were observed over time in presence of HA microparticles. In conclusion, produced HA microparticles, did not change the normal cellular behavior of primary human articular chondrocytes, in terms of viability and proliferation. Human articular chondrocytes in direct contact with HA microparticles showed a slightly increase on their chongenic somatic phenotype.

INTRODUCTION

Disability caused by joint pain is a serious problem affecting people worldwide. Pain generally results from degeneration of the joint's cartilage due to osteoarthritis (OA) or from trauma causing tissue loss. This is initially characterized by degradation and loss of cartilage tissue, hypertrophic bone changes, subchondral bone remodeling, and, at the final stage of the disease, chronic inflammation of the synovial membrane. Healthy hyaline cartilage, composed of chondrocytes embedded within a dense extracellular matrix (ECM) of collagen types II, VI and proteoglycans, such as aggrecan, has a quite low capacity to self-repair and degenerates in a one way road eventually leading to further injuries (Hunter D, 2009). Discovered over 75 years ago, hyaluronic acid or hyaluronan (HA) is a water soluble polyanion with a linear structure composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine. It plays a complex biological role due to its interactions with several HA-binding proteins, molecules of cartilage ECM as well as with cell regulating pathways of development via modulation of cellular activities. The special physiochemical and biological properties of HA and its non-immunogenic nature have made this biopolymer a particularly useful substance in biomaterials research (Roughley P, et al. 2008). In our work, we produced HA microparticles by water-in-oil emulsion technique to be applied for cartilage tissue engineering and regeneration. We analyzed the consequence of direct and non direct contact of HA microparticles on the viability and proliferation capacity of human primary articular chondrocytes. We also aimed to verify the effect of these potential microcarriers on cellular chondrogenesis commitment over time.

MATERIALS AND METHODS

Microparticles production and characterization

Hyaluronic acid microparticles were prepared using water-in-oil emulsion technique and adipic dihydrazide mediated crosslinking chemistry, adapted from Yang H,

et al. 2004. Microparticles were characterized by Scanning Electron Microscopy (SEM) and Optical Microscopy. For SEM analysis, the micrparticles were dehydrated in a series of ethanol solutions, air-dreid and then sputter coated with gold .

Human primary cell culture: isolation, expansion and *in vitro* culture

Articular cartilage biopsies were collected from patients undergoing total knee arthroplasty. Patients had signed an informed consent document approved by the Ethical Committee of at Hospital de São Marcos (Braga, Portugal). Briefly, cartilage was cleaned of connective tissue and/or subchondral bone and cut into small fragments. Individual HACs were released by repeated enzymatic digestions with collagenase I and II, Hyaluronidase and trypsin at 37 ºC. Cells were pooled, counted and seeded in multiwell plates to investigate the effect of HA micropartcles in direct and non-direct contact with HACs.

MTT assay

The viability of HACs was evaluated at different time intervals, using thiazolyl blue staining (MTT) in which absorbance data correlate to cell number. After 3 h incubation, the medium was collected and the converted dye was solubilized with 1 ml absolute ethanol. Dye absorbance was measured at 570 nm with background subtraction at 670 nm.

DNA quantification

Human articular chondrocytes proliferation for the studied contidions was determined using a fluorimetric ds DNA quantification kit. Briefly, 28.7 µL of each sample and standards were transferred to an opaque 96 well plate. Then 71.3 µL of PicoGreen solution and 100 µL of Tris EDTA buffer were added. Standards and samples were prepared in triplicate. The plate was incubated for 10 minutes in the dark and the fluorescence was measured using an excitation wavelength of 485 nm and emission of 528 nm. DNA amounts were calculated from the calibration curve.

RNA isolation and quantitative real time polymerase chain reaction (qRT-PCR)

Human articular chondrocytes at different time points were washed in PBS, immersed in Trizol reagent and kept at -80 ºC for subsequent RNA extraction. Cartilage specific markers, such collagen type I, II as well as COMP, SOX9 and aggrecan, were quantified using as reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Expression level of each target gene was calculated using the -2^{DDCt} method.

Flow cytometry analysis (FACs)

The phenotype of human articular chondrocytes cultured in polystyrene tissue culture plates in non-direct contact with HA micropatrticles was assessed by flow cytometry. Briefly, harvested cells were incubated with fluorescent monoclonal antibodies against CD105, CD44 and CD90 for 15 minutes at room temperature. Unlabeled controls were included in every experiment to evaluate the unspecific binding. Samples were analyzed using a FACScalibur with CellQuest analysis software.

RESULTS AND DISCUSSION

Microparticles with a regular circular shape and having diameters between 8 and 40 µm were obtained using our methodology. *In vitro* cultured human articular chondrocytes showed that our hyaluronic acid microparticles did not show any kind of cytotoxicity and consequent decrease on cell viability. FACS revealed non-significant changes on the expression of CD44, 90 and 105 over time. Expression of COMP, SOX9 and aggrecan (cartilage-specific genes) were upraised when in presence of HA microparticles after 7 and 28 days. Collagen type I, II were fairly constant over culture time in both conditions.

CONCLUSIONS

We verified a non cytotoxic effect of HA microparticles on articular chondrocytes cellular behavior on both studied conditions. Over time, chondrogenic gene markers were slightly upraised in presence of HA microparticles. Our results suggest that HA microparticles enhance the maintenance of the chondrogenic phenotype over time and can be used as an injectable drug carrier for cartilage regeneration.

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