Thermoreversible Hyaluronan-based Hydrogels for Cartilage Repair.

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INTRODUCTION:
Hyaluronic acid (HA) has been proposed for the replacement of damaged cartilage. Ideally, these hydrogels should be injectable and provide an appropriate matrix for chondrocytes. Recently, thermo-reversible hyaluronic acid (HA) hydrogels were synthesized by "click chemistry" and RAFT copolymerization. The properties of these hydrogels could be finely tuned and a composition suitable for cell encapsulation was identified and characterized by low viscosity at room temperature, gelling temperature ~30°C, no volume change associated with gelling and good cytocompatibility [1].

However, open questions remain relating to the ability of these hydrogels to maintain the phenotype of chondrocytes encapsulated within them and their responsiveness to mechanical load. Since mechanical stimuli play a crucial role in the development and maintenance of healthy cartilage [1], in this study, we report the effect of compressive and shear loading on the gene expression and matrix production of chondrocytes cultured in the hyaluronan-based thermo-reversible hydrogel as compared to a fibrin gel control.

METHODS:
HA-nP-NIPAM synthesis: Hydrosoluble hyaluronan propargylamide (HA-pNIPAM) was prepared from hyaluronan Streptococcus equi (HA) [Mn = 1.7 x 10^6 g.mol^-1] using an already established procedure [2]. The synthesis of azido terminated poly(N-isopropylacrylamide) (N-I-pNIPAM) with Mn equal to 20 x 10^6 g.mol^-1 was performed by RAFT copolymerization. Poly(N-isopropylacrylamide) grafted hyaluronan (HA-pNIPAM) syntheses were performed by dissolving HA in distilled water at 0.5 w/v. N-I-pNIPAM corresponding to 25% amino-propargylamine modified disaccharide subunits was added with CuSO4·5H2O and ascorbic acid sodium salt. After dialysis (MWCO = 12-14 x 10^3 g.mol^-1) against 0.1 M NaCl and water, the solutions were frozen at -80 °C and lyophilized to constant weight. Chondrocyte encapsulation: Primary chondrocytes were isolated from articular joints of 4-month-old calves. Cells were suspended in either 10% w/v HA-nP-NIPAM solution in PBS or a fibrinogen solution (30 x 10^6 cells/ml). The cell suspension was then seeded into polyurethane scaffolds (diameter 8 mm, height 4 mm, porosity 90%, pore size 300-600 μm). Five million cells were seeded on each scaffold (n = 3). A thrombin solution was added to fibrinogen-seeded scaffolds to allow gelling. After 1 hr gelling at 37°C, HA thermo-reversible and fibrin gel seeded scaffolds were transferred to a 12-well plate containing DMEM-10%FCS. After 3 days of culture, culture media was supplemented with 1% non-essential amino-acids, ascorbic acid (50 μg/ml), L-proline (40 μg/ml) and aprotinin (500 U/ml). Cells were cultured for one week in DMEM-10%FCS with medium change every 3 days. After 1 week pre-culture, one sample group was loaded for 2 weeks in a bioreactor (6 days of loading per week, 1 hr/day, 10% sinusoidal strain compression at 0.1 Hz and 25° shear at 0.1 Hz) [3], while the control group was kept in well-plates. After loading, both samples and controls were analysed for DNA (by PicoGreen assay) and GAG (by DMMB assay) contents, gene expression and histological staining. Statistical analysis: Mann-Whitney test was performed with SPSS 18 software; only p values ≤ 0.05 (*) were considered to be statistically significant.

RESULTS:
After two weeks of loading, the GAG/DNA ratio was higher for the chondrocytes cultured in the HA thermo-reversible hydrogel (17.5 ± 10.7) as compared to the fibrin (5.0 ± 3.7). The unloaded controls had a similar GAG/DNA content (6.0 ± 0.9 and 5.3 ± 1.0 for the thermo-reversible hydrogel and the fibrin, respectively). The unloaded thermo-reversible hydrogel had a lower GAG/DNA compared to the loaded one. However, the GAG retention in the scaffolds was higher in the unloaded groups as compared to the loaded ones for both gel compositions.

The extracellular matrix in the HA thermo-reversible hydrogel was mainly distributed in regions close to the polyurethane scaffold, while it was uniformly distributed in the fibrin samples (figure 1).

The loading had a positive effect on the maintenance of the chondrocytic phenotype. For both hydrogels, collagen I was down-regulated and collagen II and aggrecan were up-regulated after loading. The down-regulation of collagen I and the up-regulation of aggrecan were significantly higher in the HA thermo-reversible hydrogel than in the fibrin loaded samples. Superficial zone protein (lubricin) and hyaluronan synthase 1 were also up-regulated by loading in both gel compositions. No significant differences were found for these genes between the two types of hydrogel (figure 2).

Figure 1: Toluidine blue staining after 2 weeks of culture without load: a) HA thermo-reversible hydrogel and b) fibrin.

Figure 2: Gene expression profile of bovine chondrocytes after two weeks of loading in HA thermo-reversible hydrogel or fibrin control. Collagen I (col1), collagen II (col2), aggrecan (agg), superficial zone protein (szp) and hyaluronan synthase 1 (has1) expressions relative to unloading controls.

DISCUSSION:
This study shows for the first time the potential of mechanical stimuli on a HA thermo-reversible hydrogel for chondrocyte culture in an in vitro model. Indeed, the HA thermo-reversible hydrogel elicited a stronger response to loading than the fibrin control in terms of collagen I down-regulation and aggrecan up-regulation. The down-regulation of collagen I and the up-regulation of aggrecan are often regarded as positive markers for the maintenance of the chondrocytic phenotype and the build-up of the extra-cellular matrix. These results were confirmed by the higher GAG/DNA ratio in the loaded HA thermo-reversible hydrogel samples as compared to the unloaded control or to fibrin.

The better behaviour of articular chondrocytes in the thermo-reversible hydrogel as compared to fibrin may be due to the more appropriate visco-elastic mechanical properties of the gel as well as the more appropriate chemical composition. Indeed, several studies have shown the positive effect of hyaluronan on matrix production and prevention of de-differentiation of chondrocytes [4].

In conclusion, the HA thermo-reversible hydrogel provides an injectable carrier for articular chondrocytes that maintains their phenotype and promotes matrix synthesis. Longer-term cultures would be needed to assess the expression of these genes at the protein level, as well as the evaluation of the stability of the HA thermo-reversible hydrogel with time.

REFERENCES: