

Fine structure and molecular content of human chondrocytes encapsulated in alginate beads

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Abstract

Preservation of the chondrocytic phenotype *in vitro* requires a 3D (three-dimensional) culture model. Diverse biomaterials have been tested as scaffolds for culture of animal chondrocytes; however, to date, none is considered a gold standard in regenerative medicine. Here, we studied the fine structure and the GAGs (glycosaminoglycans) content of human chondrocytes encapsulated in alginate beads by using electron microscopy and radioactive sulfate [³⁵S] incorporation, respectively. Cells were obtained from human cartilage, encapsulated in alginate beads and cultured for 28 days. [³⁵S]Na₂SO₄ was added to the culture media and later isolated for quantification of the sulfated GAGs found in three compartments: IC (intracellular), IB (intra-bead) and EB (extra-bead). Round cells were seen isolated or forming small groups throughout the alginate. Human chondrocytes presented the features of active cells such as euchromatic nuclei, abundant RER (rough endoplasmic reticulum) and many transport vesicles. We observed an extracellular matrix rich in collagen fibres and electrondense material adjacent to the cells. Most of the GAGs produced (74%) were found in the culture medium (EB), indicating that alginate has a limited capacity to retain the GAGs. CS (chondroitin sulfate), the major component of aggrecan, was the most prominent GAG produced by the encapsulated cells. Human chondrocytes cultured in alginate can sustain their phenotype, confirming the potential application of this biomaterial for cartilage engineering.

Keywords: alginate; bioengineering; cartilage; electron microscopy; glycosaminoglycan

1. Introduction

Reduction of friction during joint movement is the most important function of the hyaline cartilage. Cartilage lesions due to traumas, diseases or aging are common challenges for orthopaedic medicine. The capacity of hyaline cartilage to self-repair is very low because of its intrinsic features such as limited cell division, lack of vascular supply and a highly elaborated ECM (extracellular matrix). The hyaline ECM is composed by a unique molecular organization (types II, VI, IX, X, XI collagens and highly hydrated aggrecan) that confers the tissue its mechanical behaviour (Hunziker and Schenk, 1984). Aggrecan is formed by numerous lateral branches of the GAGs (glycosaminoglycans) CS (chondroitin sulfate) and DS (dermatan sulfate), whose negative charges attract cations and water. Aggrecans are crucial for the absorption of compression forces applied to the joints, while type II collagen acts to limit tissue distension (Heinegård and Oldberg, 1989).

Regenerative approaches applied to cartilage lesions such as mosaicplasty, perichondrial or periosteal autografts present some limitations due to the reduced bond between the native cartilage and the transplant and the restricted amount of cartilage harvested from the donor site (Nakagawa, 2006; Hangody et al., 2008; Kessler et al., 2008). Regenerative medicine typically makes use of engineered tissues in which functional cells are seeded into

biocompatible materials and then transplanted to a target tissue (Vacanti and Upton, 1994). In cartilage engineering, chondrocytes proliferate as a monolayer in culture conditions and dedifferentiate into a fibroblast-like phenotype, resulting in a significant decrease in the synthesis of hyaline ECM molecules (Simon and Jackson, 2006). A practical method to avoid non-desirable dedifferentiation is the cultivation of chondrocytes in a 3D (three-dimensional) system using a biodegradable material as a scaffold (Marijnissen et al., 2002; Lin et al., 2009). Alginate is commonly used as a 3D scaffold to culture different cell types, including chondrocytes (Alsberg et al., 2001; De Vos et al., 2006; Dobratz et al., 2009). Alginate beads loaded with animal chondrocytes and then transplanted to cartilage lesion can sustain the hyaline morphology and biochemistry, which are important features for a successful tissue repair (Hauselmann et al., 1994). Human cells were used in this work, suggesting that the findings might be applicable to the treatment of human lesion.

2. Materials and methods

2.1. Isolation and 3D culture of human chondrocytes

Cells were isolated from articular cartilage of a healthy adult patient who underwent orthopaedic surgery. The University

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Abbreviations: 3D, three-dimensional; CS, chondroitin sulfate; cpm, counts per minute; DS, dermatan sulfate; ECM, extracellular matrix; EB, extra-bead; GAGs, glycosaminoglycans; HS, heparin sulfate; IB, intra-bead; IC, intracellular; RER, rough endoplasmic reticulum; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

Hospital Ethical Committee exempted the patient's agreement term, since the tissue would be discarded with no personal identification. Cells were enzymatically dissociated and expanded in monolayer. Mixed with 1.2% alginic acid sodium salt were 10^6 cells/ml (Sigma-Aldrich, A2033) and then dripped using a 22-gauge needle into a 102-mM CaCl_2 solution. Cells were cultivated for 28 days.

2.2.1. GAGs isolation and purification

^{35}S Na_2SO_4 , 40 $\mu\text{Ci}/\text{ml}$ was added to the culture medium to be incorporated in GAGs (Garcia-Abreu et al., 1996). $^{35}\text{SO}_4$ GAGs were analysed according to their localization: EB (extra-bead), present in the medium; IB (intra-bead), recovered after bead dissolution with 55 mM sodium citrate and IC (intracellular) after the treatment with 2% Triton[®] X-100. The obtained pellets were washed, lyophilized and weighed. The powder obtained from each fraction was incubated in 1% papain for 24 h at 60°C. $^{35}\text{SO}_4$ GAGs were purified by anion-exchange chromatography (DEAE-Sephacel), eluted with 2 M NaCl, dialysed and freeze-dried.

2.2.2. Enzymatic digestion and deaminative cleavage

$^{35}\text{SO}_4$ GAG aliquots were incubated in chondroitinase AC or chondroitinase ABC for 8 h at 37°C (Saito et al., 1968). The deaminative cleavage was performed using nitrous acid according to Shively and Conrad [34]. $^{35}\text{SO}_4$ GAGs [10000 (cpm) counts per minute] were incubated with 200 μl HNO_2 for 10 min at room temperature and finally neutralized with 1 M Na_2CO_3 .

2.2.3. Electrophoresis

GAGs were analysed by agarose gel electrophoresis according to Dietrich and Dietrich (1976). Before and after the enzymatic digestion and deaminative cleavage, $^{35}\text{SO}_4$ GAG aliquots (1000 cpm) were withdrawn from each fraction and transferred to 0.5% agarose gel for a run of 1 h at 110 V. CS, DS and HS (heparin sulfate) standards (Sigma) were added in the gels. $^{35}\text{SO}_4$ GAGs were revealed by autoradiography. Gel bands were dissolved in 0.5% PPO/toluene and the radioactivity measured (PerkinElmer, Tri-carb 2900 liquid scintillation counter).

2.3. Electron microscopy

Beads were fixed in buffered 2.5% glutaraldehyde under microwaves (Pelco LMP 3450) for two cycles of 10 s at 35°C, postfixed in 1% OsO_4 and dehydrated in ethanol. For SEM (scanning electron microscopy), the samples were critical-point dried (Balzers CPD 050), gold sputtered (BalTec 020) and observed in a Jeol SEM 5310 (10 kV) microscope. Cells, 250, from 13 fractured beads were measured using ImageJ software (NIH). The total number of cells inside the beads was calculated in two steps: (i) multiplying the volume fraction of the cells inside the beads by the bead volume, where the total volume of cells inside each bead was obtained; (ii) dividing this volume by the volume of individual cells, where the total number of cells inside the bead was obtained.

For TEM (transmission electron microscopy) studies, samples were embedded in PoliBed 812 resin (EMS), sectioned (70 nm), stained with 2% uranyl acetate and observed in a Jeol 1200EX (100 kV) microscope.

3. Results

SEM showed that the alginate beads had a drop shape (rounded with a small cusp) with a mean diameter of 1 ± 0.4 mm/dry bead (Figure 1a). Internally, the beads showed a homogeneous mesh with tiny filaments of approximately 5-nm thick (Figures 1b and 1c). We observed several cells partially entrapped on the bead surface (Figure 1d). Fractured beads revealed scattered cells throughout the scaffold (Figure 1e). Approximately 5.8×10^5 cells with a mean diameter of 8.78 ± 2.45 μm were encapsulated per bead. TEM revealed chondrocytes alone or in small groups covered by spherical structures similar to exocytic vesicles (Figure 1f, inset) or by fibrillar materials (Figure 1g). Groups formed by two up to eight cells within the lacunae were also seen (Figures 1f and 1h). Human chondrocytes presented euchromatic nuclei with dispersed chromatin, abundant RER (rough endoplasmic reticulum), many mitochondria and numerous transport vesicles (Figures 1h and 1i). Often, groups of cells were seen lining a central compartment containing ECM molecules (Figure 1h). We also observed abundant ECM associated with the cells and dispersed throughout the beads (Figure 1i). High magnification of the ECM revealed amorphous materials with different densities and many collagen fibres (Figures 1i and 1j).

Electrophoresis of the ^{35}S GAGs recovered from the culture medium (EB) revealed two bands: a large band composed of chondroitin and dermatan and a second one of heparan (Figure 2). Enzymatic treatments confirmed the GAG composition. ^{35}S GAGs recovered from the IB region showed two bands: chondroitin and heparan. For the IC fraction, we observed a major band composed of chondroitin (Figure 2). $^{35}\text{SO}_4$ counts indicated that 73.8% of GAGs was located from the EB, where chondroitin was the predominant glycan (Table 1). Dermatan was only detected in the EB compartment. A similar amount of chondroitin and heparan was detected from the IB, while chondroitin was the major glycan found inside the cell (Table 1).

4. Discussion

The 3D culture is not only a functional model to investigate cellular metabolism, ultrastructure and gene expression, but also a cell delivery method for bioengineering. The biomaterial used as scaffold behaves as a semi-rigid medium, allowing the chondrocytes to receive appropriate mechanical stimuli. Chondrocytes express some integrin receptors with high affinity for ECM molecules, such as type II collagen and fibronectin, playing a critical role in the phenotype maintenance (Genes et al., 2004). The appropriate activation of those integrin receptors may explain the phenotype preservation when chondrocytes are cultured in a 3D environment.

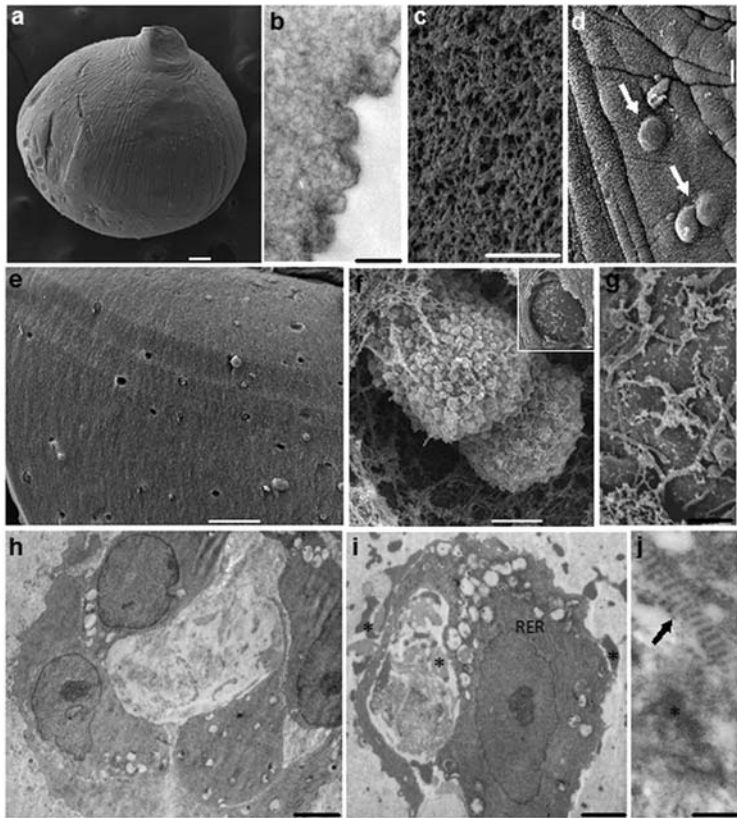


Figure 1 Morphology of the alginate beads and chondrocytes

(a) SEM image showing an alginate bead with a drop shape (rounded with a small cusp). Bar, 100 µm. (b) TEM image of the bead periphery showing the arrangement of alginate fibres. Bar, 1 µm. (c) SEM of a fracture bead revealing the mesh aspect formed by alginate fibres. Bar, 1 µm. (d) SEM of a bead surface showing round cells partially entrapped by the alginate (arrows). Bar 10 µm. (e) SEM of a fractured bead showing the cells distribution inside the scaffold. Bar 100 µm. (f) Two chondrocytes covered by ECM material resembling exocytic vesicles. Bar, 5 µm. (f) Inset, single chondrocyte embedded in the alginate. Bar, 1 µm. (g) Detail of the cell surface showing the fibrillar material. Bar, 0.5 µm. (h) TEM of a cell group forming a central space filled with ECM. Bar, 3 µm. (i) TEM of a chondrocyte showing its typical ultrastructure: prominent nucleus with dispersed and granulated chromatin, abundant RER and numerous transport vesicles. Cell-associated extracellular matrix surrounding a cell and dispersed through the alginate mesh can be seen (*). Bar, 2 µm. (j) Detail of a collagen fibre with their typical banding pattern (arrow) and ECM dense materials (*). Bar, 0.1 µm.

Alginate as a biomaterial to deliver chondrocytes can be a promising therapeutic method for cartilage lesions (Dobratz et al., 2009). Noteworthy, the alginate used should be carefully chosen because of its potential antigens and molecular variation.

Alginates are mostly isolated from brown algae and can still contain many impurities after the chemical extraction. Orive et al. (2002) showed that the total purification of the alginate dramatically reduced the impurities (63% less of polyphenols, 91.5% of

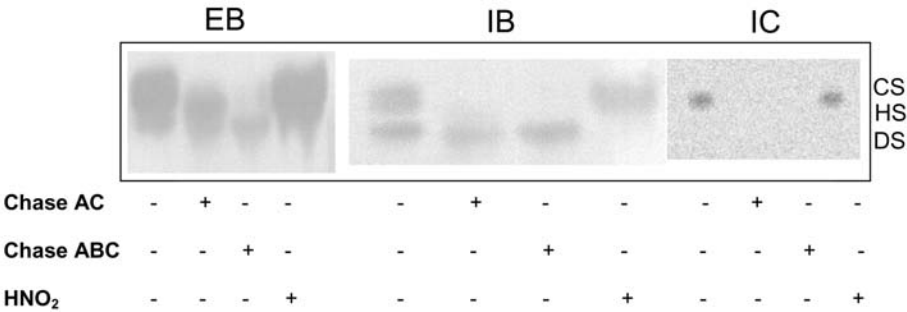


Figure 2 Agarose gel electrophoreses of ³⁵S-GAGs recovered from the culture media or EB, IB and IC

The results of the GAGs treatment with the enzymes chondroitin AC lyase, ABC and nitrous acid are shown below the gel. Plus (+) sign represents a positive enzymatic reaction. In EB, the bands correspond to CS and DS. GAGs from IB have two major bands composed of HS and CS. In the IC fraction, a major band of CS was seen.

Table 1 [³⁵S] counts of CS, HS and DS detected in the three studied regions

Numbers in bold represent the percentage of total cpm, while numbers in italic represent the percentages of CS, HS, DS. Nd, non-detectable.

Structure	Total cpm (%)	Chondroitin sulfate	Heparan sulfate	Dermatan sulfate
Extra-bead	27696 (73.86)	4142 (<i>61.3</i>)	540 (<i>8</i>)	2067 (<i>30.6</i>)
Intra-bead	5554 (14.83)	410 (<i>57</i>)	386 (<i>49</i>)	nd
Intracellular	4235 (11.31)	1532 (<i>90.2</i>)	210 (<i>8.8</i>)	nd

endotoxins and 68.5% of proteins) and prevented antibody response against alginate beads implanted in mice. Considering the alginate backbone, Wong et al. (2001) showed that alginates formed by high guluronic concentration had maximum values for dynamic modulus compared with the mid and low guluronic amounts, indicating that for the best mechanical integrity, high guluronic alginates are preferable.

We have shown by TEM that ECM molecules, such as collagen fibres and amorphous materials, were produced by the encapsulated human chondrocytes. [³⁵SO₄]GAG assays indicated that the alginate could not retain those ECM molecules inside the beads, since the highest amount of GAGs was measured outside the beads. Carboxyl groups present in alginates are directly linked with calcium ions (egg-box model) used to form the jellified beads. Since the free carboxyls are the active sites, ECM molecules would be loosely attached to the alginate mesh and consequently are more mobile. We detected high concentrations of chondroitin, a key molecule in the aggrecan formation. Even though the alginate cannot retain the majority of the produced GAGs, we could speculate that the loss of ECM molecules could accelerate the neoformation of cartilage around the implanted bead.

Based on the cellular ultrastructure, human chondrocytes encapsulated in alginate for 28 days proved to be healthy with active metabolism. In addition, we did not observe dead or apoptotic cells in the centre of the beads indicating that oxygen and nutrients could diffuse throughout the beads. Carossino et al. (2007) used alginate-polylysine beads to encapsulate human chondrocytes from elderly patients in order to proliferate and preserve the phenotype. These authors showed by TEM the presence of viable well-granulated cells with the typical morphological aspects of adult chondrocytes, such as oval-shaped cells forming clusters, prominent nuclei, abundant RER, mitochondria and lysosomes. Here, we observed groups of human cells within the alginate, resembling the typical isogenous group formations found in natural cartilage.

Cartilage bioengineering has been considered one of the most promising treatments for human lesions. We showed that a commercially available alginate can sustain chondrocyte phenotype, although only medical-grade alginate must be chosen to encapsulate the cells to be transplanted into the patient's lesion. It is a consensus that the patient's own cells should be used in the therapy to avoid implant rejection. So far, monolayer culture is the most used method for cell proliferation prior to implant transplantation. Our results show the efficient establishment of a 3D culture of human chondrocytes into a biomaterial compatible for human use. More studies should be done to develop the cultivation methods for transplantaion and applications in regenerative medicine.

5. Conclusions

3D culture using alginate beads contributes to the recovery of the chondrocyte phenotype usually lost during monolayer expansion. Human chondrocytes encapsulated in alginate beads were healthy and show an active metabolism, since GAGs and collagen fibres normally found in the ECM of cartilage were produced.

Author contribution

Leonardo Rodrigues Andrade wrote the paper. Leonardo Rodrigues Andrade, Katia Denise Arcanjo, Humberto Saramago Martins and Maria Eugenia Leite Duarte designed the research. Leonardo Rodrigues Andrade, Katia Denise Arcanjo, Humberto Saramago Martins and João Sergio Nobre dos Reis performed the research. Leonardo Rodrigues Andrade, Katia Denise Arcanjo, Humberto Saramago Martins, João Sergio Nobre dos Reis, Marcos Farina, Radovan Borojevic and Maria Eugenia Leite Duarte analysed data.

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