ENCAPSULATION AND GROWTH CHARACTERISTICS OF THREE DIFFERENT CELLS IN ALGINATE GEL BEADS CONTAINING RECONSTITUTED COLLAGEN FIBERS

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ABSTRACT

Alginate gel beads containing reconstituted collagen fibers were fabricated and used to culture three different cells - GH3 pituitary tumor cells, L929 fibroblasts and rat calvaria osteoblasts. These cells when entrapped within the collagen-containing alginate behaved differently from those grown in the alginate matrix. Of the three cells tested, the existence of collagen fibers is most beneficial for the viability and proliferation of GH3 cells which can grow either as suspended or attached to the matrix. For the case of osteoblasts derived from the primary culture of rat calvaria, most of the cells entrapped in alginate lost their viability after seeding. The collagen fibers in the alginate gel beads nevertheless decelerated the apoptosis process. Furthermore, the entrapped osteoblasts were capable of migrating out of the collagen-containing alginate matrix when the alginate gel beads were in contact with the culture dish. The mineralization of matrix, a key phenotypic expression of osteoblastic cells, was shown by von-Kosa staining on the culturing dish after 14 days cultivation of the cells which crawled out of the gel beads. The collagen-containing alginate beads appear to be more versatile than the conventional alginate gel beads for biomedical applications.

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1. INTRODUCTION

Alginate has been used frequently to entrap and

culture cells for industrial or biomedical applications.[1-2] The entrapped cells are surrounded by the alginate molecules, which bear negative charges at physiological pH. Many reports have pointed out that mammalian cells did not adhere well to a negatively charged surface. Alginate is a copolymeric polysaccharide of D-Mannuronic acid and L-Guluronic acid, thus, contains a significant number of negative

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charges at physiological pH. This is a major reason why adherent cells, when entrapped in the alginate gel beads, could not migrate in the alginate matrix and their growth was inhibited in this environment.[3]

Fibrous collagen is a major component in the connective tissues, and has been utilized in many biomedical applications. Many cells need to attach to the collagen fibers for migration, differentiation and proliferation.[4-5] Therefore, gel beads consisting of a matrix of fibrous collagen are potentially useful for culturing cells of various types.

In lieu of the important applications of alginate gel in constructing scaffolds of tissue engineering, we have developed a method of preparing gel beads containing collagen matrix.[6] We now report our experimental results of using this technique to entrap and culture three different cells. The growth characteristics and morphologies of the entrapped cells - GH3, L929 and osteoblast cells, are discussed in this paper.

2. MATERIAL AND METHOD

Material

Alginate (from *Macrocystis pyrifera*) was obtained from Kelco (a division of Merck Co.). Type I collagen was prepared according to the following procedure. The rat-tail tendon was soaked in 0.5M acetic acid for 3-4 days at 4°C. The extract was then incubated in 0.5M acetic acid containing 0.5 mg/ml of pepsin for 24 hours. The insoluble portion was removed from the digest by centrifuge at 12,000 rpm (JA14, Beckman) for one hour at 4°C. Salt was then added to the pepsin-treated extract to a final concentration of 1 M NaCl and incubated for overnight. After centrifuged at 12,000 rpm, the precipitate was collected and resuspended in 0.5M acetic acid. Finally, the collagen solution was dialyzed against 0.5M acetic acid at 4°C to remove salt. The collagen solution was stored in 0.5M acetic acid at 4°C.

Isolation of Osteoblast Cells

Osteoblasts were prepared according to the method of Nefussi⁸ with slight modification. Briefly, calvaria from neonatal Sprague-Dawley rats were excised aseptically. After removing the endo- and extra-cranial periosteum, the calvaria was minced into fragments of approximately 1 mm³. The bone fragments were treated with collagenase (1mg/ml) at 37°C for 2 hrs. After centrifugation at 1500 rpm for 5 min, the supernatant was collected. The cell pellet thus obtained was then resuspended in DMEM supplemented with 10% fetal calf serum, and incubated at 37°C under the atmosphere of 5% CO₂. For

identifying osteoblast, cells were fixed with glutaraldehyde and then stained by a mixture of Naphthol-AS-Mx phosphate sodium salt and fast blue BB salt.

Encapsulation of Cells in Collagen-containing Alginate

Before used, the stocked collagen solution was dialyzed against 0.02M PBS (pH 7.2) and centrifuged at 48,000 rpm (Ti70.1, Beckman) for 3 hours. The upper two thirds of the solution containing soluble collagen was collected from the centrifuge tube. The collagen solution was mixed with alginate and cells to attain the final concentrations of approximately 1.3mg/ml collagen, 1.2w/v alginate and cell density of 10⁵ cells/ml. The mixture was extruded from a syringe through a 23G cannula into 1.5% CaCl₂ (pH 7.2) to form the spherical gel beads. Air was applied extracannally at a rate of 5 l/min to control the size of gel beads. The gel beads were collected and washed 4 min with Ca-CHES buffer (1.1% CaCl₂ in 0.1% 2-N-cyclohexylamino ethane-sulfuric acid, pH 7.2). After that, they were treated with an aqueous solution of 0.1%, poly(L-lysine) for 2min and then washed repeatedly with Na-CHES. The gel beads were resuspended in 50 mM sodium citrate, pH 7.2, for 2 min, then washed with Na-CHES and resuspended in PBS (0.15 M NaCl in phosphate buffer, pH7.2). Collagen reconstitution was conducted by incubating the gel beads at 37°C for 2 hr. The encapsulation of cells in alginate gel beads was conducted by the above procedure without the addition of collagen and the gel beads were coated subsequently with polylysine and alginate to stabilize the gel beads.

The above cell-containing collagen/alginate (or alginate as control) gel beads were suspended in 4 mL of DMEM (Dubecco's Modified Eagles Medium) and incubated at 37°C under the atmosphere of 5% CO₂. At various times after incubation, the gel beads were treated with collagenase (0.5 mg/ml) and citrate for 5 min to solubilize the matrix and detach the cells from collagen fibers. For the case of microencapsulation, microcapsules (derived from 500ul) were disrupted by micro centrifuge sample pestle, mixed with 250ul collagenase/250ul citrate and incubated for 30 minutes at 37°C to release the cells from the matrices. The number of the viable cells was determined by trypan blue exclusion method.

3. RESULT AND DISCUSSION

Both collagen and alginate are soluble and miscible to each other in the ice-cold aqueous solution at the neutral pH. Alginate, in the presence of

collagen, still maintained its Ca^{+2} -induced gelling capability to form spherical gel beads. On the other hand, the monomeric collagen triple helices maintain their capability of reconstitution into fibrous network regardless the intermixing alginate molecules when the temperature was raised to 37°C . We further investigated the microstructures of surface and inner core of the gel beads. The reconstituted collagen fibers existed in both surface and the inner region of the gel beads. However, it appeared that a significant amount of alginate remained inside the gel bead. The collagen fiber reconstituted in alginate is smaller but with the same morphology as those reconstituted in the absence of alginate. Albeit the difference in the sizes of collagen fibers, alginate seems to have minimal effect on the reconstitution process of collagen fibers.

When GH3 rat pituitary tumor cells were entrapped in the collagen-containing gel beads, they grew from 1.5×10^5 cells/ml to about 2×10^6 cells/ml after 12 days of incubation (Figure 1). In contrast, the GH3 cells maintained at a very low density when entrapped and cultured in the alginate gel beads. The appearance of the GH3 cells in the alginate/collagen gel beads is very similar to that on the hydrated collagen matrix. As shown in Figure 2a and 2b, the cells appeared in round shape with many spikes extruding from the cell surface. The cells attached to collagen fibers and sometimes appeared to be wrapped inside a group of interpenetrated collagen fibers. On the other hand, GH3 cells spread and flatten on the plastic surface when cultured on petri-dish (Figure 2c). Thus the collagen-containing gel beads provide an environment more closely related to the hydrated collagen matrix as compared to tissue culture plate.

Fig 1. Growth curve of GH3 cells in alginate gel beads containing collagen fibers (●-●), and in alginate gel beads (-▲-▲).

Fig 2a. SEM micrograph of GH3 cells grown on collagen matrix on the 4th day after seeding.

Fig 2b. SEM micrograph of GH3 cells grown within a collagen-containing alginate gel bead as observed on the 9th day after encapsulation.

Fig 2c. SEM micrograph of GH3 cells grown on culture glass as observed on the 3rd day after seeding.

We then applied this technique to entrap L929 fibroblast cells in the collagen-containing gel beads. The growth curve of these cells is shown in Figure 3. As shown, the cell density increased from 3.5×10^4 cells/ml to 8.7×10^4 cells/ml after 6 days of incubation. The morphology of the cells attached to the collagen fibers was examined under a confocal microscope. As shown in Figure 4, L929 fibroblasts had their pseudopods attached along the fibers. In addition, the fibroblasts had a round shape when not in contact with the collagen fiber in the matrix of gel beads. It appeared that there are many fragments of unsolubilized alginate co-existed with reconstituted collagen fibers in the gel beads. Only when in contact with the collagen fibers, 3T3 fibroblasts were capable of spread and proliferated.

We further employed the encapsulation system to entrap the osteoblast cells prepared from rat calvaria and studied their growth profiles. Different from the cultured cells of GH3 and L929, the number of the viable osteoblast cells (primary culture) entrapped within the alginate decreased dramatically immediately after cell seeding. The cell density declined to about 2×10^4 cells/ml on the 4th day after encapsulation and remained at this low level throughout the experiment. It appears that the existence of collagen fibers in alginate matrix reduces the apoptosis rate of the entrapped osteoblast cells, and the resulted cell density was double that in the alginate matrix without collagen. As shown, the density of osteoblasts decreased gradually from 1.4×10^5 to 3×10^4 cells/ml in

11 days when cultivated in the collagen-containing gel beads (Figure 5). When the osteoblast-containing collagen/alginate gel beads were gently disrupted by a pestle and placed in the culture flask, the osteoblast cells migrated from the gel beads onto the flask surface and proliferated. On the other hand, the osteoblasts were unable to migrate onto the surface of culture dish if they were entrapped within the matrix consisted of

Fig 4. A fibroblast cell entrapped and grown within the collagen-containing alginate gel beads observed under confocal microscope. The fibril attachment (arrow) of the fibroblast cells can be clearly seen under the microscope.

Fig 3. Growth curve of L929 cells in alginate gel beads containing collagen fibers (●-●-), and in alginate gel beads with layer coatings of polylysine and alginate (-▲-▲-).

Fig 5. Growth curve of osteoblast cells in collagen-containing alginate gel beads (-▲-▲-), and in alginate gel beads (●-●-).

Fig 6. Micrograph of matrix mineralization by osteoblast cells migrated out of the collagen-containing alginate gel beads onto culture dish and process for von Kosa stain.

alginate only. The osteoblast cells probably migrated from the gel beads along the collagen fibers onto the dish surface. Figure 6 shows the microscopic photograph of matrix mineralization by the osteoblasts migrated from the collagen-containing alginate gel beads and cultured for another 12 days on petri-dish. The inorganic deposit by the mineralization process of osteoblast cells can be clearly seen on the dish surface after von Kosa staining.

In this study, we have prepared collagen-containing alginate gel beads and investigated its applications in the culturing of adherent cells. Three different types of cells were entrapped and showed different growth characteristics within the collagen-containing microspheres. The results demonstrated that the collagen-containing gel beads provide a cell type-selective specific growth support, and may have potential applications in the areas of biomedical implant and biochemical process.

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