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ScienceDirect
Journal of Biotechnology 130 (2007) 75–84
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

Engineering cartilage tissues with the shape of human nasal alar by using chondrocyte macroaggregate—Experiment study in rabbit model

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Received 4 December 2006; received in revised form 26 February 2007; accepted 27 February 2007

Abstract

Despite of progresses in tissue engineering based on cell/scaffold strategy, uneven cell distribution as well as tissue formation in the scaffold, limited cell seeding efficiency and inflammatory reaction triggered by the degradation of scaffold remain problems to be resolved. In this study, we proposed a novel cell–macroaggregate cultivation system, and explored a feasible strategy to construct three-dimensional cartilage tissue with shape of human nasal alar by using cell macroaggregate. Isolated chondrocytes was cultured at high density to form a monolayer chondrocyte sheet as well as expanded for seeding on the sheet to produce mechanically operable cell macroaggregate. Chondrocyte macroaggregates were then fabricated into transplants with shape of nasal alar by using Internal support or External scaffold techniques; results of in vivo chondrogenesis were investigated in immunocompetent animal. Chondrocyte macroaggregates presented long survival time and good viability; constructs fabricated using both techniques can develop into tissues with characteristic structure of native cartilage, glycosaminoglycans as well as type II collagen were highly produced in the ECM of engineered cartilages. By placing hyaluronan ester film as Internal support, the predetermined shape of the chondrocyte macroaggregate can be well maintained. In contrast, due to the poor mechanical stability of grafts fabricated in External scaffold group, obvious deformation occurred in harvested specimens. The experiment proved the usefulness of chondrocyte macroaggregate in cartilage regeneration, and provided a new strategy to engineer cartilage with special shape by using cell macroaggregate/biodegradable support.

Keywords: Tissue engineering; Chondrocyte macroaggregate; Nasal alar; Cartilage; Internal support

1. Introduction

Cartilage repair remains an obstacle in clinical works because of poor regenerative capacity of cartilage tissues and limitation of donor sites. As an important technique for cell transplantation, tissue engineering provided a new concept for tissue regeneration and reconstruction, which has been expected to regenerate autogenic cartilage grafts by combining isolated cells and scaffold with predetermined shape (Cao et al., 1997). Over the past decade, various scaffolds including collagen, chitosan, silk protein, and synthetic degradable polymers have been developed to support the growth of chondrocytes isolated from various animal species (Hutmacher, 2001; Woodfield et al., 2002). However, these scaffolds have their respective problems including mechanical strength, cell dedifferentiation, inflammatory reaction induced by their degradation products and limited cell seeding efficiency (Sittinger et al., 2004; Britt and Park, 1998).

Strategies using natural reaggregation potential to assemble monodispersed cells in a tissue-mimicking way represent a valuable extension of current scaffold-based tissue engineering initiatives (Risbud and Sittinger, 2002). Okano et al. developed a temperature responsive culture dish, which can be used to harvest cultured cells non-invasively as intact sheets along with their deposited extracellular matrix (ECM). By transplanting monolayer or layered cell sheets, encouraging results in engineering functional myocardial patches, urothelium tissue and cornea have been achieved (Joseph et al., 2005). The principle advantage of cell sheet is that an entirely natural tissue assembled by cells, with mature ECM, can be engineered, which avoids shortcomings in scaffold based design. However, a principal drawback of cell sheet is their poor mechanical properties, which

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doi:10.1016/j.jbiotec.2007.02.029

makes it hard to be fabricated into grafts with special shape, size and controllable stiffness (Ng and Hutmacher, 2006).

For cartilage tissue engineering, fabricating cartilage grafts is of great significance for reconstructive surgery in head and neck region, and three-dimensional shape of cartilage graft must be taken into consideration to achieve cosmetic contour. In addition, chondrocytes will lose their differentiated phenotype during in vitro expansion, which leads to less production of glycosaminoglycans and type II collagen (Brodkin et al., 2004; Thirion and Berenbaum, 2004). Previous studies (Benya and Shaffer, 1982; Van Osch et al., 2001; Schulze-Tanzil et al., 2002) have demonstrated that three-dimensional culture and high-density cultivation will facilitate the redifferentiation of passaged chondrocytes. Naumann et al. (2004), developed a three-dimensional in vitro macroaggregate culture system by using a cell culture insert, which allowed expanded chondrocytes to maintain good phenotype and form homogeneous cartilage graft which approximates the clinical size for cartilage defects in nose and auricle. However, due to the poor mechanical stiffness, the grafts lack stable shape to meet the need of reconstructive surgery.

Given the above, we have explored the use of a new culture system, which aimed to fabricate cell macroaggregate composed of cultured chondrocytes and self-produced ECM. Then chondrocyte macroaggregates were used to engineer cartilage tissues with special shape (nasal alar) by using "Internal support" and "External scaffold" techniques. We hypothesized that the combination of chondrocyte macroaggregates and Internal biodegradable support could provide grafts with better mechanical stability as compared with macroaggregate fabricated by External scaffold, which may facilitate the cartilage formation with special shape in immunocompetent animals. Rabbits were used to evaluate the cartilage formation in the shape of nasal alar by two different constructing approaches.

2. Materials and methods

2.1. Animal model

Twelve New Zealand rabbits (2-month-old) were used for the experiment. The operative procedure and the care of the rabbits were performed under the regulation of Experimental Animal Centre, Fourth Military Medical University. The rabbits were acclimated for 1 week before operation and monitored for general appearance, activity, excretion and weight, then they were randomly divided into two groups: External scaffold group ($n = 6$) and Internal support group ($n = 6$).

2.2. Isolation and culture of chondrocytes

All the New Zealand rabbits were anesthetized with ketamine (40 mg/kg, i.m.) and xylazine (5 mg/kg, i.m.). After aseptic preparation, auricular cartilage from ear roots was dissected and minced into about 2 mm^3. After being rinsed three times with phosphate buffered saline (PBS) supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL), the cartilage samples were digested with 0.2% collagenase type II (Gibco) in Dulbecco's Modified Eagle Medium (DMEM; Gibco) at 37 °C for 12 h. The digested cell suspension was filtered through a 250 μm nylon mesh filter to remove matrix debris and was centrifuged at 1000 rpm for 5 min, the resulting cell pellet was washed two times with PBS and resuspended for cell seeding. Cell counting was performed with hemocytometer and cell viability was assessed by trypan-blue exclusion test.

Isolated chondrocytes were divided into two parts, one-third of them were plated in a culture dish ($d = 6$ cm) at density of $1.5–2.0 \times 10^6$ cells/dish for the production of chondrocytes-matrix membrane (chondrocyte sheet, Step 1, Fig. 1a), DMEM-high glucose culture medium (Gibco) containing 15% fetal bovine serum, L-glutamine (272 μg/mL), ascorbate 2-phosphate (50 μg/mL) and transforming growth factor-beta (TGF-β, 10 ng/mL, R&D) was added, this medium is referred to as "medium I". The other two thirds of primary chondrocytes were routinely cultivated in T flasks. To enhance the proliferation of chondrocytes, DMEM-low glucose culture medium supplemented with 10% fetal bovine serum (Hyclone), L-glutamine (272 μg/mL, Sigma), ascorbate 2-phosphate (50 μg/mL, Sigma) and 10 ng/mL basic fibroblast growth factor (b-FGF, Cell concepts) was added (Step 1, Fig. 1a), this medium is referred to as "medium II" in the following. Cells were expanded for 3 passages and medium was changed every 3 days during cell culture.

2.3. Culture strategy for generation of chondrocyte macroaggregate

After being cultured for three passages, expanded chondrocytes were collected with 0.25% trypsin/EDTA solution (Gibco) and centrifuged to remove digestion solution, then "medium I" was added to resuspend amplified chondrocytes. On the average, about 6.0×10^7 cells per sample were allowed to deposit by gravity onto the formed chondrocyte sheet (Step 2, Fig. 1a). Afterward, the cell macroaggregate was incubated in 5% CO_2 with 100% humidity at 37 °C for 5 days, and "medium I" was changed every 2 days.

During the high-density cultivation, the chondrocytes further secrete white substances on the bottom of the dish, which enhanced mechanical stability of macroaggregate. At the 5th day after cell-seeding, a solid white membrane formed and can be easily detached from the bottom of the dish with cell scraper. For the examination of the microscopic structure, three pieces of chondrocyte macroaggregate were dissected and taken out for further processing. One piece was transferred into PBS containing 2 μM CFDA (Sigma) and incubated for 10 min at 37 °C, then was taken out and rinsed twice with PBS to process for observation by confocal microscope. The second piece was fixed, dehydrated, embedded in paraffin and sectioned for Safranin-O staining. The third piece was processed for transmission electronic microscope (TEM) examination.

2.4. In vitro fabrication of three-dimensional transplants

The chondrocyte macroaggregate was detached completely from the culture dish with a cell scraper and fabricated according to the following approaches (Fig. 1b, Step 2).