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Experimental Study on Self-assembly of KLD-12 Peptide Hydrogel and 3-D Culture of MSC Encapsulated within Hydrogel *In Vitro*[*]

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Summary: To synthesize KLD-12 peptide with sequence of AcN-KLDLKLDLKLDL-CNH_2 and trigger its self-assembly *in vitro*, to encapsulate rabbit MSCs within peptide hydrogel for 3-D culture and to evaluate the feasibility of using it as injectable scaffold for tissue engineering of IVD. KLD-12 peptide was purified and tested with high performance liquid chromatography (HPLC) and mass spectroscopy (MS). KLD-12 peptide solutions with concentrations of 5 g/L, 2.5 g/L and 1 g/L were triggered to self-assembly with 1×PBS *in vitro*, and the self-assembled peptide hydrogel was morphologically observed. Atomic force microscope (AFM) was employed to examine the inner structure of self-assembled peptide hydrogel. Mesenchymal stem cells (MSCs) were encapsulated within peptide hydrogel for 3-D culture for 2 weeks. Calcein-AM/PI fluorescence staining was used to detect living and dead cells. Cell viability was observed to evaluate the bioactivity of MSCs in KLD-12 peptide hydrogel. The results of HPLC and MS showed that the relative molecular mass of KLD-12 peptide was 1467.83, with a purity quotient of 95.36%. KLD-12 peptide at 5 g/L could self-assemble to produce a hydrogel, which was structurally integral and homogeneous and was able to provide sufficient cohesion to retain the shape of hydrogel. AFM demonstrated that the self-assembly of KLD-12 peptide hydrogel was successful and the assembled material was composed of a kind of nano-fiber with a diameter of 30–40 nm and a length of hundreds of nm. Calcein-AM/PI fluorescence staining revealed that MSCs in KLD-12 peptide hydrogel grew well. Cell activity detection exhibited that the *A* value increased over the culture time. It is concluded that KLD-12 peptide was synthesized successfully and was able to self-assemble to produce nano-fiber hydrogel *in vitro*. MSCs in KLD-12 peptide hydrogel grew well and proliferated with the culture time. KLD-12 peptide hydrogel can serve as an excellent injectable material of biological scaffolds in tissue engineering of IVD.
Key words: peptide; self-assembly; biological scaffolds; three-dimensional cell culture

The degeneration of intervertebral disc (IVD) is a chronic pathological process involving a variety of factors. Pathologically it is characterized by apoptosis of nucleus pulposus cells, decreased water content and proteoglycan[1–3]. Recently, tissue engineering methods have been introduced to the early intervention for this condition and some encouraging results have been achieved. As an important component of tissue engineering of IVD, biological scaffolds have been a subject of keen investigation. KLD-12 peptide hydrogel is a new self-assembling material and has been studied as a skeleton for the repair of articular cartilage defect[4]. The researches on the KLD-12 peptide have been scanty and use of KLD-12 peptide hydrogel as cell skeleton for tissue engineering of IVD has not been reported. In this study, we synthesized KLD-12 peptide, triggered its self-assembly *in vitro* and then encapsulated rabbit mesenchymal stem cells (MSCs) with the hydrogel for *in vitro* three-dimensional (3-D) culture. The purpose of the study is to evaluate the feasibility of employing the KLD-12 as cell skeleton for tissue engineering of IVD.

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1 MATERIALS AND METHODS

1.1 KLD-12 Peptide Molecule

KLD-12 peptide with sequence of AcN-KLDLKLD-LKLDL-CNH_2 (K: lysine; L: leucine D: aspartic acid) was synthesized by using a peptide synthesizer (Shanghai Jier Biological Technology Co., China) and lyophilized to powder, which was purified and tested with high performance liquid chromatography (HPLC) and mass spectroscopy (MS).

1.2 Isolation and Culture of Rabbit MSCs

Three-month old New Zealand white rabbits with average weight of about 2 kg, were purchased from Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (License No. SCXK(ER) 2004-0007). Animals were subjected to gen-

eral anesthesia with 10% chloral hydrate. Then 4-mL bone marrow aspirates were taken from the greater trochanter of rabbit femur and diluted with L-DMEM. MSCs were isolated with a Percoll density gradient centrifugation[5] and re-suspened in complete culture medium (*L*-DMEM) supplemented with 10% FBS. The cells were plated into a T-75 culture flask with a cell density $5\times10^6/cm^2$ and incubated at 37°C, in 5% CO_2. Half of the culture medium was changed after 48 h and then the culture medium was changed once every three days. When the monolayer cultures have grown to approximately 90% confluence, MSCs were treated with 0.25% trypsin for 5 min at 37°C, then re-plated into a T-75 flask and incubated for about 4 days. The third generation of cells (P3) was harvested and stored for later use.

1.3 Self-assembly of KLD-12 Peptide Hydrogel

KLD-12 powder (10 mg) was dissolved in distilled water and then lightly vortexed for 30 min and KLD-12 peptide with final concentration of 5 g/L, 2.5 g/L and 1 g/L was prepared. KLD-12 solution (400 μL) of 5 g/L, 2.5 g/L and 1 g/L were respectively put into a small bottle, and triggered into self-assembling with 1×PBS solution for 20 min at 37°C. Afterwards, PBS solution was discarded, and the shape of assembled material was grossly observed.

1.4 Atomic Force Microscope (AFM) test

KLD-12 peptide self-assembled hydrogel (0.1 g/L) was oscillated for 30 min. Then a drop of 1 μL was placed on the mica sheet of AFM, washed twice using 100-μL distilled water and air-dried for 5 h. AFM was used to detect its inner nanofibers.

1.5 The 3-D Culture of MSCs Encapsulated within KLD-12 Peptide Hydrogel

KLD-12 powder (10 mg) was dissolved in 10% sucrose solution at a peptide concentration of 0.56% (w/v). MSCs (P3) were re-suspended in a complete culture medium, and 0.56% peptide solution was added to obtain peptide/cells suspension, with a final peptide concentration of 0.5% and a cell density of 20×10^6/mL. The peptide/cell suspension was lightly vortexed and injected into the wells as previously described by Kisiday *et al*[4] and triggered into self-assembling with 1×PBS solution for 20 min at 37°C. Then, complete culture medium (*L*-DMEM supplemented with 10% FBS) was added to the wells containing peptide/cells hydrogel, and the well plates were transferred into an incubator with saturated humidity at 37°C in 5% CO_2) for two weeks. The culture medium was changed regularly, and the cells were morphologically observed under an invert microscope.

1.6 Calcein-AM/PI Fluorescein Staining

KLD-12 peptide solution (300 μL, 0.56%) was added to 48-well plates. MSC encapsulation within KLD-12 peptide hydrogel and self-assembling were the same as described above. The culture medium was changed once every two days. Seven and 14 days after the culture, cells encapsulated in the hydrogel of 4 wells were taken for Calcein-AM/PI fluorescein staining. Briefly, in a light-avoided environment, 100 μL of Calcein-AM/PI (Calcein-AM: 2 μmol/L, PI: 4.5 μmol/L) was added, to each well, and the cells were then incubated for 20 min at 37°C. Cells were observed under an inverted fluorescein microscope for viability. Under blue excitation light, the living cells showed green color and the nucleus of dead cells exhibited red fluorescence.

1.7 Cell Viability Evaluation

KLD-12 peptide solution (100 μL, 0.56%) was added to 96-well plates. MSCs were encapsulated within KLD-12 peptide hydrogel and self-assembling was triggered by using above-mentioned technique. The peptide/cells hydrogel cultured for different time lengths was designated as experimental groups, and the 0.5% peptide hydrogel without cells served as control groups. The culture medium was changed once a day. After 1-, 3-, 7- and 14-day culture, samples were taken from 6 wells from each group for cell viability evaluation. CCK-8 (10 μL) was added to the wells, which were then incubated for 4 h at 37°C and detected for the absorbance (*A* value) by using a universal microplate spectrophotometer (wavelength of excitation light: 490 nm).

1.8 Statistical Analysis

The data were expressed as $\bar{x}\pm s$ and processed with the SPSS12.0 software package. Data were analyzed by using the independent-samples *t*-test. And a $P<0.05$ was considered to be statistically significant.

2 RESULTS

2.1 Detection of KLD-12

KLD-12 peptide was synthesized successfully. The relative molecular mass was 1467.83, with a purity quotient of 95.36%, which was proved by HPLC and MS.

2.2 KLD-12 Self-assembling

KLD-12 peptide solution (5 g/L) could be self-assembled to produce a hydrogel, which was structurally integral and homogeneous and had sufficient cohesion to retain the shape of hydrogel. But the self-assembled hydrogel of KLD-12 with concentration of 2.5 g/L and 1g/L was heterogeneous, with only few hydrogel granules formed in some areas (fig. 1).

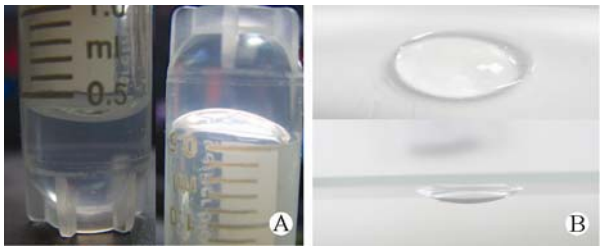

Fig. 1 The self-assembled KLD-12 peptide hydrogel of 5 g/L
A: KLD-12 peptide hydrogel in small bottle: The hydrogel outline was homogeneous and did not have obvious fluidity when the bottle was inverted.
B: KLD-12 peptide hydrogel on glass slide: The hydrogel outline was homogeneous without remarkable gel particles found in the drop. There was some elasticity on the surface. The hydrogel did not have obvious fluidity when the slide was inverted.

2.3 AFM

AFM showed that the self-assembly of KLD-12 peptide hydrogel was successful and the assembled ma-

terial was composed of a kind of nanofiber with a diameter of 30–40 nm and a length of hundreds of nm. These nanofibers in KLD-12 peptide hydrogel were interwoven to form a net-like structure with pores (fig. 2).

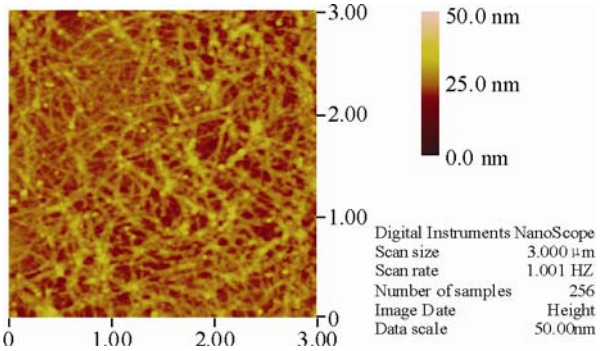

Fig. 2 The self-assembled KLD-12 peptide hydrogel as detected by AFM

The assembled material was a kind of nanofiber with diameter of 30–40 nm and length of hundreds of nm. These nanofibers interwove and form a porous network structure

2.4 Calcein-AM/PI Fluorescein Staining

At the 7th day, the MSCs in KLD-12 peptide hydrogel maintained its round shape and the cells at the bottom of hydrogel wells presented spindle or fusiform shape. Some MSCs proliferated and were of dumb-bell shape. A lot of living cells were found in peptide hydrogel, with few dead cells. At the 14th day, the cells in peptide hydrogel grew well. Compared with the cells at the 7th day, cell morphology remained unchanged, but cell proliferation was more apparent (fig. 3).

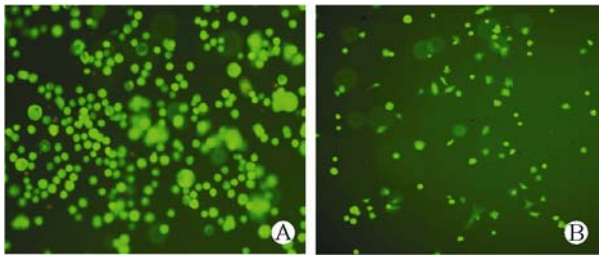

Fig. 3 The morphology of MSCs in KLD-12 peptide hydrogel by Calcein-AM/PI fluorescein staining at the 7th days

A: The MSCs in KLD-12 peptide hydrogel maintained round shape. MSCs in KLD-12 peptide hydrogel grew well. Few of dead MSCs was detected (red color).

B: Some MSCs at the bottom of peptide hydrogel are of spindle or fusiform shape.

2.5 Cell Viability

The *A* value increased gradually with the culture time. Compared with control groups, the *A* value of each experimental group was significantly increased ($P<0.05$). There were statistically significant differences in *A* value among the experimental groups ($P<0.05$) (fig. 4).

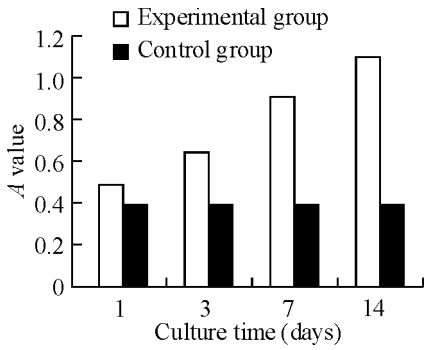

Fig. 4 Cell viability ($n=6$)

The *A* value increased gradually with the culture time. Compared with control groups, the *A* value of each experimental group was significantly increased ($P<0.05$). There were statistically significant differences in *A* value among the experimental groups ($P<0.05$).

3 DISCUSSION

Lumbar vertebral degeneration is believed to be the main cause of back pain[6]. With the increase of age, degenerative lumbar IVD undergoes the morphological changes, suffers from metabolic disorder[7, 8], with water and proteoglycan content of nucleus pulpous decreased[1, 9]. Nowadays, tissue engineering technology has been introduced into the treatment for early IVD degeneration. The core of tissue engineering of IVD research is cell scaffolds and seed cells. The ideal scaffolds should have geometry, porosity and good biocompatibility, maintain cell phenotype, enhance cell proliferation and extracelluar matrix (ECM) production[10–12]. They also should be biodegradable along with endogenic matrix production. Some scaffolds such as alginate gel[13–16] and collagen scaffold[17–21] have been proved to be effective as support for implanting cells into nucleus pulposus. These scaffolds, however, lack structural integrity[22]. Recently, self-assembling peptide hydrogel as a kind of new injectable material is used for tissue repair. Essentially, its molecular structure consists of amino acids, and self-complementary oligopeptides contain 50% charged residues. The hydrophilicity and hydrophobicity amino acid residues alternate and repeat. Self-assembling peptide hydrogel is easy to modify. Moreover, with the self-assembling peptide hydrogel, self-assembling is easy to trigger and encapsulation of seed cells was convenient *in vitro*. KLD-12 is a type Ⅰ peptide, with repeating sequence of 4 amino acids and an arrangement of electric charges in the form of "+ – + – + –", and presents as β-sheet structure in water. Kisiday *et al*[4], for the first time, designed and synthesized KLD-12 peptide molecule and used it to encapsulate chondrocytes for 3-D culture *in vitro*. The experimental results showed that the chondrocytes encapsulated within the KLD-12 peptide hydrogel retained their phenotype and produced proteoglycan and type Ⅱ collagen. The hardness of KLD-12 peptide hydrogel increased with the accumulation of the ECM.

The self-assembly and feasibility of using KLD-12 peptide hydrogel as cell skeleton in the tissue engineer-

ing of IVD have not been reported. In our study, the KLD-12 peptide was synthesized and examined using HPLC and MS. The relative molecular mass of KLD-12 peptide was 1467.83, which was identical to theoretical molecular mass, and the purity quotient was 95.36%. The results proved that KLD-12 peptide was synthesized successfully. The *in vitro* experiment showed that KLD-12 peptide at 5 g/L could self-assemble to form the integral hydrogel when triggered by PBS. The self-assembled peptide hydeogel had enough cohesion to maintain the shape, and the surface of hydeogel had some elasticity. AFM showed that, ultrastruturally, the nanofibers self-assembled by the peptide were 30–40 nm in diameter and hundreds nm in length. The fibers interwove to a porous structure. The porous scaffold can facilitate the diffusion of nutrients and accumulation of ECM.

MSCs are ideal seed cells for tissue engineering of IVD. It has been proved that MSCs could differentiate into cells with nucleus pulposus-like phenotype when induced by exogenous biological factors *in vitro*[5, 23], which is the theoretical basis for using MSCs as seed cells in tissue engineering of IVD. Sobajima *et al*[24], proved that MSCs could significantly promote the ECM production of nucleus pulposus cells (NPCs) *in vitro*. In their study, allogenic MSCs were implanted into rabbit IVD, and 24 weeks after the implantation, the histological examination showed that those transplanted MSCs survived well and integrated into the inner annulus fibrous of rabbit discs, and MSCs were able to exert therapeutic effects on IVD degeneration through autocrine and paracrine mechanisms. Le Maitre *et al*[25] implantated MSCs into bovine caudal IVD, and their postoperative observation over a period of 28 days demonstrated that the transplanted allogenic MSCs differentiated into cells with a nucleus pulposus-like phenotype. More studies proved that MSCs have promoting effect on regeneration of IVD. We selected MSCs as seed cells and KLD-12 peptide as scaffold for tissue engineering of IVD. Rabbit MSCs were encapsulated within KLD-12 peptide hydrogel for 3-D culture for 2 weeks *in vitro*. Fluorescence staining of Calcein-AM/PI showed that the cells in peptide hydrogel grew well and maintained round shape and the cells at the bottom of hydrogel were of spindle or fusiform shape. At the 7th day, some MSCs proliferated and showed dumbbell shape and at the 14th day, cells did not experience apparent morphological changes, but the number of proliferating cells increased significantly. Detection of cells viability showed that the *A* value increased gradually over culture time. There were significant differences in the *A* value among the experimental groups of different time periods ($P<0.05$), suggesting that the viability of the MSCs in peptide hydrogel increased with time. The possible reason might be that the cells proliferated in the peptide hydrogel. Our study proved that MSCs encapsulated within KLD-12 peptide hydrogel grew well.

To sum up, KLD-12 peptide was synthesized successfully and was able to self-assemble to produce nanofiber hydrogel *in vitro*. The MSCs in KLD-12 peptide hydrogel grew well and proliferate with culture time. It is concluded that KLD-12 peptide hydrogel is an excellent injectable biological scaffold material for tissue engineering of IVD.

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