Construction of tissue engineered cartilage *in vivo* with poly(lactide-co-glycolic acid) composited with collagen II and growth factors☆☆☆★△

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Abstract

BACKGROUND: The development of cartilage tissue engineering provides novel ideas for treatment of articular cartilage defects and implements construction of tissue-engineered cartilage *in vivo*.

OBJECTIVE: To investigate the feasibility of constructing tissue-engineered osteochondral composite through bone marrow stem cells(BMSCs) cultured on the poly(lactide-co-glycolic acid) (PLGA), which was modified with collagen and cellular growth factors.

METHODS: PLGA was made by phase separation technique, composited with collagen II, basic fibroblast growth factor, and transforming growth factor-β1. The BMSCs of passage 3 were cultured on the above scaffolds. Thirty-six SD rats were randomly divided into experimental, control, and blank groups. These three groups received implantation of BMSCs composited with growth factors and collagen-PLGA, implantation of BMSCs composited with collagen-PLGA, and implantation of collagen-PLGA into the muscle, respectively. At 4, 8, and 12 weeks after surgery, cell directional differentiation and growth were examined by gross observation, hematoxylin-eosin staining, toluidine blue staining, collagen II staining, and scanning electron microscope.

RESULTS AND CONCLUSION: Gross observation showed that there were many chondroid tissues in the experimental group and fibrous tissues in the control and black groups. Stainings and electron microscope revealed that many chondroblasts and a few osteoclasts appeared in the composite of the experimental group. Toluidine blue and collagen II stainings were positive in the experimental group and negative in the control and blank groups. These findings demonstrate that PLGA modified with collagen had a good cellular compatibility. BMSCs cultured on PLGA, which was modified with collagen and cellular growth factors, can construct the tissue-engineered osteochondral composite in rats.

INTRODUCTION

Nowadays, the methods to repair articular cartilage defects remain limited, and all traditional treatments can not achieve satisfactory results[1-2]. The development of cartilage tissue engineering provides novel ideas for treatment of articular cartilage defects. Cartilage tissue engineering requires proper scaffold, sufficient normal seed cells, and cell factors that can regulate cell proliferation and keep cell phenotype characteristics[3]. According to this principle, this study was designed to construct tissue-engineered osteochondral composite through bone marrow stem cells(BMSCs) cultured on the poly(lactide-co-glycolic acid) (PLGA), which was modified with collagen and cellular growth factors, then implanted this composite into rats' muscle. By observing the construction condition of tissue-engineered osteochondral composite *in vivo*, we intended to explore a better method to construct the tissue-engineered osteochondral composite, hopefully better repairing articular cartilage defects.

MATERIALS AND METHODS

Design

Randomized, controlled, animal experiment.

Time and setting

Performed at the Central Laboratory, First Affiliated Hospital of Jinan University between November 2008 and August 2009.

Materials

Thirty-six Sprague-Dawley rats of SPF grade, weighing approximately 200 g, were provided by Guangdong Laboratory Animal Research Institute. All experimental protocols were in accordance with Animal Ethical Standard.

The primary reagents and instruments used in this study are as follows:

Reagent/instrument	Source
PLGA	Jinan Maikang, China
Collagen II, transform growth factor-β1 (TGF-β1)	Sigma, USA
Bone morphogenic protein-2 (BMP-2), basic fibroblast growth factor (bFGF)	Bioengineering Research Institute of Jinan University, China
Dimethyl benzene, wax, hematoxylin, eosin, dioxane	Guangzhou Chemical Factory, China
Scanning electron microscope	Hitachi, Japan
Type EKF030 drying vacuum oven, lyophilizer	Shanghai Reagent and Instrument Factory, China

Methods

Preparation of composite scaffolds

Preparation of collagen II-modified PLGA: PLGA (70 : 25) was dissolved in dioxane, and was agitated uniformly for 3 hours. Then the resultant solution was left on flat plate overnight at −20 ℃, placed into the

alcoholic solution (volume percentage was 30 : 70) for three extractions, each 8 hours. All cake samples were freeze-dried in the lyophilizer, and then cut them into suitable sizes (10 mm × 10 mm × 5 mm). 5 mg collagen II was added into 5 mL phosphate buffered saline for 1 hour of agitation. Ten PLGA scaffolds were placed into the mixture and followed by additional 1 hour of agitation. Following vacuum lyophilization, collagen II-modified PLGA was harvested.

Preparation of PLGA composited by bFGF, TGF-β1 and collagen II: ①PLGA was prepared as above. ②10 mg bFGF was added into 5 mL PBS containing 5 mg collagen II for 1 hour of agitation. ③10 μg TGF-β1 was added into 5 mL PBS containing 5 mg collagen II for 1 hour of agitation. ④Ten PLGA samples prepared in step ① were respectively added into step ② for 1 hour of agiation. ⑤The composite obtained in step ④ was dried in the lyophilizer. ⑥ Ten PLGA samples composited with bFGF and collagen II were added the mixture prepared in step ③ for 1 hour of agiation. ⑦ The composite prepared in step ⑥ was dried in the lyophilizer to harvest PLGA composited with bFGF, TGF-β1 and collagen II.

Seeding BMSCs

The BMSCs of passage 3 were adjusted to 10^9/ L. Following sterilization by ^{60}CO and DMEM washes as well as drying, PLGA scaffolds composited with bFGF, TGF-β1 and collagen II were placed into the holes of 24-well plate. 100 μL cell suspension was added for 1 hour incubation. After that, PLGA scaffolds were reversed and additional 100 μL cell suspension was added for 1 hour culture[4].

Construction of cartilage complex tissue and observation of related data

Thirty-six SD rats were randomly divided into experimental, control, and blank groups. These three groups received implantation of BMSCs composited with growth factors and collagen-PLGA, implantation of BMSCs composited with collagen-PLGA, and implantation of collagen-PLGA into the muscle, respectively. At 4, 8, and 12 weeks after surgery, cell directional differentiation and growth were examined by gross observation, hematoxylin-eosin staining, toluidine blue staining, collagen II staining, and scanning electron microscope.

Following intraperitoneal anesthesia (pentobarbital 1 μg/kg), gluteus maximus muscle was bluntly separated *via* a posterolateral incision made on the leg to construct muscle bag. Composite scaffolds were implanted into the muscle bag according to grouping situations, followed by suture of sarcolemma, fascia, and skin. Feeding was continued.

Main outcome measures

Gross observation: At 4, 8, and 12 weeks after surgery (n = 4), the construction of cartilage complex tissue was observed in terms of luster and rigidity.

Hematoxylin-eosin staining: the samples were fixed with 10% neural formaldehyde, decalcificated with routine acidic EDTA decalcification solution, dehydrated with ethanol, cleared with xylene, embedded with paraffin, sliced(horizontal and vertical direction continuously, thickness 5 μm), and dyed with hematoxylin-eosin for observation of sample structure.

Toluidine blue staining: Following fixation by 40 g/L paraformaldehyde, the composite tissue was dehydrated in a gradient ethanol series, embedded in organic resin, sliced with histotome (in horizontal and vertical directions continuously, thickness 5 μm), stained with toludidine blue for optical microscope observation.

Collagen II staining: following fixation by 40 g/L paraformaldehyde, samples were dehydrated in gradient ethanol series, embedded in organic resin, sliced (horizontal and vertical direction continuously, thickness 5 μm). Then rabbit anti-rat collagen II monoclonal antibody (1: 200) as primary antibody and goat anti-rabbit SABC as secondary antibody were added. Diaminobenzidine was used as the chromogenic agent. According to the standard instruction of SABC kit, immunohistochemistry was performed, followed by optical microscope.

Scanning electron microscope observation: At 12 weeks after surgery, samples were fixed in 2% glutaradehyde for 24 hours, fixed at -20 ℃, and then dried with a vaccum dryer. After that, the samples were plated with gold and observed by scanning electron microscopy.

Design, enforcement and evaluation

The first, second, third, and fourth authors designed this study. The first, third, fourth, and fifth authors collected data. The first, fifth, and tenth authors performed experimental procedures. The first, sixth, and ninth authors evaluated experimental data, and blind method was used.

RESULTS

Gross observation

4 weeks after surgery

In the experimental group, scaffold material shape changed, and a little scaffold material had been absorbed. Some chondroid tissue appeared and had a weak hardness. In the control group, similar symptoms were observed with exception of chondroid tissue. In the blank group, structural morphology changed greatly, more materials had been absorbed, texture was soft, and no changes in color were observed.

8 weeks after surgery

In the experimental group, ivory white chondroid tissue was much more than before, and felt much harder when touching. In the control group, a little white area could be seen, and this area felt hard, but no chondroid tissue formed. In the blank group,

Xiong GX, Zha ZG, Tan WC, Wu H, Li JR, Lin HS, Xia JS, Huang XP, Tu M, Zhang JQ. Construction of tissue engineered cartilage in vivo with poly(lactide-co-glycolic acid) composited with collagen II and growth factors. Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu. 2010;14(16): 3028-3032.

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the material surface was covered by a layer of compact fibrous tissue. Material structure had a much greater change in shape, felt quite soft when touching, and no chondroid tissue could be observed.

12 weeks after surgery

In the experimental group, ivory white chondroid tissue grew much larger thanbefore. In the control group, material surface felt hard, but no chondroid tissue was observed. In the blank group, a little fibrous tissue could be seen on the material surface, and most scaffold material had been absorbed, and felt quite soft.

Hematoxylin-eosin staining

4 weeks after surgery

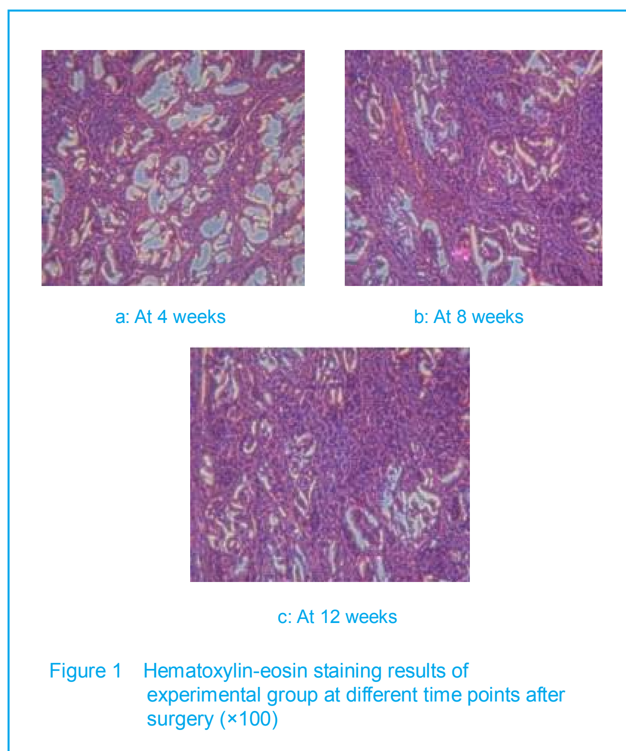
In the experimental group, the PLGA had been degraded into net-like structure, a lot of shuttle-like stem cells could be seen, and many round and oval-shaped chondroblasts grew into the gap and contained much intercellular substance of chondroblasts (Figure 1a). In the control group, there were also many shuttle-shaped stem cells, but on chondrocytes were observed. In the blank group, only a few lymphocytes were seen.

8 weeks after surgery

In the experimental group, only a little net-like PGLA remained, shuttle-shaped stem cells were reduced, polygonal chondrocytes greatly increased, a little cartilage tissue formed, some multinuclear osteoclasts with much cartilage matrix were observed (Figure 1b). In the control group, a few shuttle-shaped stem cells but no chondroid cells with regular shape were observed, a part PLGA was filled with bone matrix. In the blank group, quite a few inflammatory cells were observed.

12 weeks after surgery

In the experimental group, the cells were matured chondrocytes, large osteocartilage tissue had formed, and multinuclear osteoclasts increased (Figure 1c). In the control group, only some shuttle-shaped stem cells, but no matured chondrocytes, were observed. In the blank group, many cells like inflammatory cells could be observed, and the rest part was still filled with fibrous tissue.



a: At 4 weeks b: At 8 weeks

c: At 12 weeks

Figure 1 Hematoxylin-eosin staining results of experimental group at different time points after surgery (×100)

Toluidine blue staining

4 weeks after surgery

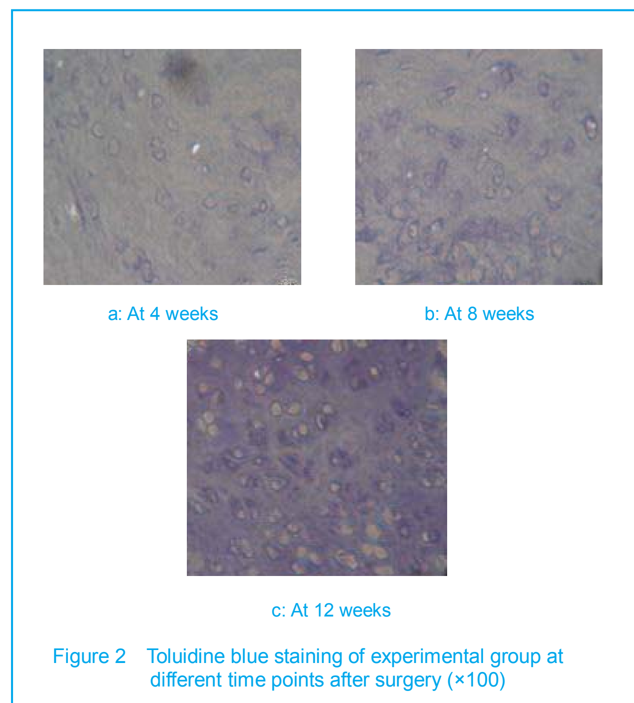
In the experimental group, the PLGA had been degraded into net-like structure. Toluidine blue staining showed that a few round or oval cells could be observed, some cells contained two nucleoli, and the reaction results were weakly positive (Figure 2a). In the control and blank groups, no chondroid cells were colored, and Toluidine blue staining results were negative.

8 weeks after surgery

In the experimental group, net-like PLGA reduced more than before, toluidine blue staining showed a large number of round or oval cells, cartilage-like substance was observed surrounding the stained nuclei, and the staining results were positive (Figure 2b). In the control and blank groups, a small amount of fibrous tissue-like tissue, but no chondroid cells, was observed, and toluidine blue staining results were negative.

12 weeks after surgery

Almost all net-like PLGA disappeared. Toluidine blue staining revealed that a large number of round or oval cells were colored, there were many cartilage matrix-like substances surrounding the stained nuclei, a small amount of woven cartilage close to mature state formed, and toluidine blue staining results were strongly positive (Figure 2c). In the control and blank groups, no chondrocytes and cartilage tissue were observed, cells were not colored, and staining results were negative.



a: At 4 weeks b: At 8 weeks

c: At 12 weeks

Figure 2 Toluidine blue staining of experimental group at different time points after surgery (×100)

Collagen II staining

4 weeks after surgery

In the experimental group, the PLGA had been degraded into net-like structure, staining results showed that some round or oval cells were colored, a few collagen II-stained cells occasionally contained two nucleoli, and collagen II staining results were weakly positive (Figure 3a). In the control and blank groups, no chondroid cells were stained.

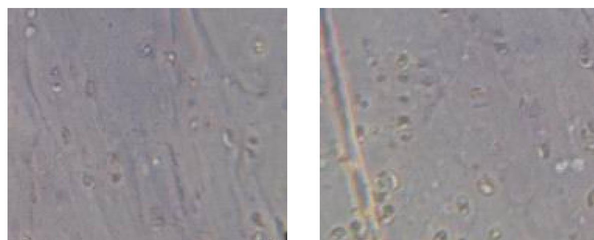
8 weeks after surgery

In the experimental group, net-like PLGA in the cartilage area reduced more than before, collagen II staining revealed that a large number of round or oval cells were colored, with a little

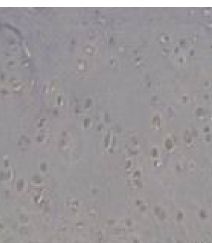
cartilage matrix-like substance surrounding the stained nuclei (Figure 3b). In the control and blank groups, a small amount of fibrous tissue-like tissue was observed, but no chondroid cells were observed, collagen II staining results were negative.

12 weeks after surgery

In the experimental group, net-like PLGA in the cartilage area almost disappeared, collagen II staining revealed that a large number of round or oval cells were colored, much cartilage matrix-like substance surrounded the stained nuclei, a small amount of woven bone similar to matured state formed, and staining results were strongly positive (Figure 3c). In the control and blank groups, no chondrocytes and cartilage tissue were observed, and cells were not stained.



a: At 4 weeks b: At 8 weeks



c: At 12 weeks

Figure 3 Collagen II staining of experimental group at different time points after surgery (×100)

Scanning electron microscope observation

At 12 weeks after surgery: In the experimental group, many shuttle-shaped stem cells were surrounded by some round or oval cells, which had tide edge and chondrocyte-like morphology (Figure 4). In the control group, there were a few shuttle-shaped cells and many polygonal and multinuclear cells, but no large number of round and oval cells with regular cell edge similar to experimental group. In the blank group, several micronuclear cells similar to inflammatory cells were observed, but no shuttle-shaped, polygonal, or multinuclear cells were observed.

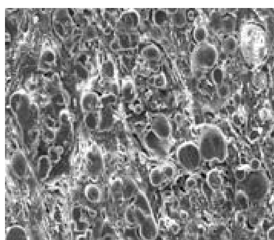


Figure 4 Scanning electron microscope observation of experimental group (× 300)

DISCUSSION

Choice of seed cells

BMSCs can be differentiated into chondrocytes and form cartilage tissue when cultivated in the presence of proper inducers. Canine BMSCs can be differentiated into chondrocytes by the induction of growth factors *in vitro*[5]. It has been reported in the literature that after separation culture and digestion passage of allogenic chondrocytes, the antigen is reduced, but the matrix-secreting function is kept once been implanted. Considering cellular segregation and reducing immune response to implanted material to the most[6], we chose autologous stem cells composited with materials and implanted the composite. Results showed that simple scaffold could be hardly differentiated into chondrocytes without seed cells. By contrast, scaffold material composited with BMSCs could be differentiated into chondrocytes, even cartilage tissue induced by growth factor *in vivo*. In addition, results also revealed that the degradation of PLGA was faster in the experimental group than in the control group or blank group. Maybe the induction of numerous BMSCs could promote the osteoclast migration by its chemotaxisduring differentiation. These findings indicate that BMSCs are ideal seed cells in cartilage tissue engineering.

Preparation of scaffold and the choice of cell factor

Cartilage tissue-engineered scaffold materials consist of inorganic and organic materials. The inorganic materials exhibit good biocompatibility, proper biodegradability, and excellent conductibility[7]. Above all, PLGA is the most common material to construct porous scaffold, because its degradation products *in vivo* are harmless to the tissue and cells[8] and the degradation rate of PLGA can be controlled by adjusting the proportion of copolymer (PLA/PGA) and relative molecular mass[9]. PLGA has been presently proved to be an ideal scaffold because of its good biocompatibility and safety[10]. The scaffold of PLGA in this study was made by extraction at low temperature and lyopyilization, in this way, it can be fabricated into the shapes we desired, which facilitates cell factor loading, with the porosity of scaffold over 85%. These characteristics are beneficial for the BMSCs growing on the scaffold and the directional differentiation into chondroblasts.

At present, scholars pay more attention to bFGF and TGF-β1 and that these two factors had comparable effects in promoting BMSCs to differentiate into chondroblasts[11-12]. bFGF can not only promote chondrocyte proliferation, differentiation, and maturation but also stimulate DNA and proteoglycan syntheses. So bFGF is both mitogen and morphogenic factor to chondroblasts[13]. Appropriate concentration of bFGF can keep active differentiation of chondrocytes and synthesis of extracellular matrix. It can also inhibit terminal differentiation and calcification[14].

Most importantly, bFGF may make fibroblasts matured or transformed into chondrocytes. Considerable studies have demonstrated that TGF-β1 is one of the essential conditions that induce BMSCs to differentiate into chondrocytes[15]. TGF-β1 can keep the stability of chondrocyte phenotype. It plays different roles in different stages of cartilage growth. TGF-β1 positively regulates Sox9 and collagen II gene expressions and promotes BMSCs differentiation into chondrocytes[16]. At the same time, it can also inhibit the differentiation of matured chondrocytes and matrix

synthesis. It also has inhibitory effect on ALP activity[17]. In the present study, we chose bFGF and TGF-β1 as cell factors in cartilage tissue engineering. Results demonstrated that bFGF and TGF-β1 could differentiate BMSCs into chondrocytes, even forming cartilage tissue *in vitro* or *in vivo*.

Features of tissue-engineered cartilage composite preparation

Collagen II added in PLGA has been confirmed to have the ability to stimulate chondrocyte reproduction and collagen deposition and simultaneously lighten the inflammation[18]. During the process of PLGA preparation, collagen was compounded and growth factors were added. We succeeded in inducing BMSCs differentiation into chondrocytes, and by the induction *in vivo*, cartilage tissue formed. The tissue-engineered cartilage collagen has the function to regulate the release of growth factors. Because bFGF and TGF-β1 were both grinded into powder, mixed with collagen, and then added into the scaffold, and before been released, there was an extended process of solution, and this process also has the function to regulate the release of growth factors. The composite of the experimental group had a significant quality of cartilage induction. By using BMSCs as the seed cells of tissue-engineered cartilage, collagen II as the vector of growth factors, bFGF and TGF-β1 as the induction factors, and PLGA as the scaffold, it is feasible to construct novel tissue-engineered cartilage composite tissue.

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聚乙醇酸-乳酸共聚物复合Ⅱ型胶原和生长因子体内构建组织工程软骨***★△

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摘要

背景：软骨组织工程的发展为处理关节软骨损伤提供了新的思路和方法，使体内构建组织工程软骨得以实现。

目的：观察骨髓基质干细胞种植到复合胶原和生长因子的聚乙醇酸-乳酸共聚物(poly-lactide-co-glycolic acid，PLGA)生物材料，再种植到大鼠体内构建组织工程软骨组织的可行性。

方法：相分离法制作PLGA，复合Ⅱ型胶原和碱性成纤维细胞生长因子，转化生长因子β1。将第3代的骨髓基质干细胞种植到复合材料上。36只SD大鼠随机分为实验组、对照组、空白组，分别于肌袋内植入骨髓基质干细胞/复合生长因子和胶原的PLGA、复合生长因子和胶原的PLGA、复合胶原的PLGA，于术后第4，8，12周取材观察细胞的定向分化及生长情况，包括大体观察、苏木精-伊红染色、甲苯胺蓝染色、Ⅱ型胶原染色、扫描电镜观察。

结果与结论：大体观察可见实验组材料有类软骨样组织生长，而对照组和空白组则仅见纤维组织生长。各种染色及电镜观察显示：实验组复合物内可见多的成软骨细胞及少量的破骨细胞。实验组甲苯胺蓝染色和Ⅱ型胶原染色为阳性，对照组和空白组均为阴性。从而证明胶原修饰的PLGA生物材料具有较好的细胞相容性；骨髓基质干细胞种植到复合胶原和生长因子的PLGA生物材料上在大鼠体内可构建组织工程软骨复合组织。

关键词：骨髓基质干细胞；聚乙醇酸-乳酸共聚物；软骨向诱导；复合组织；组织工程

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