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Regeneration of a Cartilage Tissue by *In Vitro* Culture of Chondrocytes on PLGA Microspheres

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Abstract Cartilage tissue engineering has emerged as an alternative approach for reconstruction or repair of injured cartilage tissues. In this study, rabbit chondrocytes were cultured in a three-dimensional environment to fabricate a new cartilaginous tissue with the application of tissue engineering strategies based on biodegradable PLGA microspheres. Chondrocytes were seeded on PLGA microspheres and cultured on a rocking platform for 5 weeks. The PLGA microspheres provided more surface area to adhere chondrocytes compared with PLGA sponge scaffolds. The novel system facilitated uniform distribution of the cells on the whole of the PLGA microspheres, thus forming a new cartilaginous construct at 4 weeks of culture. The histological and immunohistochemical analyses verified that the number of chondrocytes and the amount of extracellular matrix components such as proteoglycans and type II collagen were significantly greater on the PLGA microspheres constructs as compared with those on the PLGA sponge scaffolds. Therefore, PLGA microspheres enhanced the function of chondrocytes compared with PLGA sponge scaffolds, and thus might be useful for formation of cartilage tissue *in vitro*.

Key words: Cartilage, chondrocytes, tissue engineering, PLGA microspheres

Cartilage defect is a serious healthcare problem to people of all ages [2, 3, 6, 15]. Recently, tissue engineering has been considered as an attractive approach for cartilage replacement. Cartilage tissue engineering techniques generally require the use of a porous scaffold that serves a three-dimensional microenvironment for the seeded cell attachment and expansion, and subsequent tissue formation both *in vitro* and *in vivo* systems.

Various natural and synthetic scaffolds such as porous sponges, fibers, and hydrogels have been prepared from biodegradable polymers for cartilage tissue engineering [14, 16, 17, 20]. Natural biopolymers such as collagens have the potential advantages of specific cell interactions and easy seeding of cells because of their hydrophilicity [11, 13, 19, 22]. However, the natural scaffolds are mechanically unstable and do not uniformly distribute the cells on the entire scaffolds, thus they have limitations on the application of an engineered tissue. The synthetic biopolymers such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and poly (DL-lactic-co-glycolic acid) (PLGA) are biocompatible with high porosity and have the same shape as the tissues to be replaced. Moreover, synthetic scaffolds have sufficient mechanical strength, enough to retain until the formation of a new tissue *in vivo* [7, 9, 10, 12]. The disadvantages of the synthetic polymers are, however, the lack of cell-recognition signals that results in insufficient cell adhesion, and hydrophobicity that hinders uniform seeding of sufficient cell mass on the surface of the polymers. A number of hybrid forms of synthetic scaffolds have been developed to compensate for the disadvantages [1, 4, 5, 8, 18, 21, 23]. Many reports have recently published the successful fabrication of cartilage tissue *in vitro* [1, 8, 10–13, 18]. However, fabricating cartilage by the tissue engineering techniques in the reports showed some limitations on mimicking natural cartilage in terms of construct size, histologic and biochemical compositions, and sequentially mechanical property. Many things are attributed to a scaffold because it should support physical and biological behaviors to the cells, which is often contradictory to each other. In addition, the limitation of mass transfer depending on the construct volume is also a serious problem. It is due to the

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barrier formed by coating at the surface and prevents a cartilaginous tissue from being formed in the core region of the scaffold. For these reasons, fabrication of bigger size scaffolds is still limited.

To resolve the above problems, this study suggested a new method for fabrication of an articular cartilage. An articular cartilage using poly (DL-lactic-co-glycolic acid) (PLGA) microspheres was prepared and the functions of chondrocytes cultured on PLGA microspheres were evaluated.

Materials and Methods

Isolation of Chondrocytes

Chondrocytes were isolated from the knee articular cartilage of New Zealand white rabbits (Joong-Ang Experimental Animal Center, Seoul, Korea) by collagenase digestion as described previously [21]. In brief, male rabbits weighing 250 g were put into euthanasia by overdose of Nembutal injection, and non-fibrillated articular cartilage of the knee was removed by sterile dissection. Cartilage was finely minced and washed three times in phosphate-buffered saline (PBS) solution. Fragments were sequentially digested in 0.2% collagenase (Worthington Biochemical: Lakewood, NY, U.S.A.) in PBS solution for 3 h at 37°C. The digestion solution was filtered through a cell strainer (70 μm Nylon: Falcon: Franlkin Lake, NJ, U.S.A.) to remove undigested matrix. The released cells in the supernatant were collected by centrifugation (1,700 rpm, 15 min) and washed twice with PBS solution. The cell number and viability were determined by using the trypan blue (0.25%, w/v) dye exclusion method. The cells were then suspended in DMEM (Gibco BRL: Grand Island, NY, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (FBS: Gibco BRL), 100 U/ml penicillin G and 100 μg/ml streptomycin (Gibco BRL), and seeded in tissue culture flasks at a cell density of 1.5×10^5 cells/cm^2. Cells were incubated in humieified atmosphere at 37°C and 5% CO_2. The culture medium was changed every 3 days and the chondrocytes after two passages were used for the following experiments.

Preparation of PLGA Scaffolds

Poly(DL-lactic-co-glycolic acid) PLGA 75:25 microspheres with a molecular weight of 100-kDa were prepared by a solvent evaporation technique in oil-in-water emulsion. Briefly, 4 g of PLGA was dissolved in 50 ml of dichloromethane. This solution was poured into 600 ml of deionized water containing 2% (w/v) of PVA under agitation with a rate of 1,000 rpm by a mechanical stirrer for 10 min. Then, the suspension was gently stirred at 35°C for 2 h with a magnetic stirrer to evaporate the dichloromethane. The produced microspheres were collected by centrifugation at 3,000 rpm for 3 min, extensively washed four times with deionized water, and lyophilized. PLGA sponges were prepared and cultured after seeding of the cells as a control [21]. The scaffolds were sterilized and hydrated in ethanol, and rinsed three times with PBS solution. The sterilized scaffolds were then immersed in DMEM for 24 h before cell seeding.

Measurement of the Degradation of PLGA Polymer

To investigate the degradation of PLGA polymer, PLGA microspheres (10 mg) were placed in 5 ml of PBS solution in tubes and incubated on a rocking platform set at 43 cycles per minute and at an angle of 15° in a humidified incubator at 37°C and 5% CO_2 for 24 days. The degradation of the polymer was observed by SEM analysis.

Chondrocyte Seeding and Culture on the Scaffolds

The chondrocytes were collected and suspended in culture medium at a density of 1×10^7 cells/ml. One ml of cell suspension was mixed with 10 mg of PLGA microspheres and cultured on the rocking platform set at 43 cycles per minute and at an angle of 15° in a humidified incubator at 37°C and 5% CO_2 for 5 weeks. The medium was changed every day. Chondrocyte-seeded PLGA microspheres were harvested after 2 and 4 weeks for analysis. Chondrocyte-seeded PLGA sponges were cultured in the same condition as a control, as mentioned above.

Scanning Electron Microscopy Analysis

The polymeric surfaces of chondrocyte-seeded PLGA scaffolds were observed by scanning electron microscopy (SEM). Chondrocyte-seeded PLGA scaffolds were fixed using 4% (w/v) glutaraldehyde solution for 24 h, and washed three times in PBS solution to remove residual fixative. The samples were then dried using a graded series of ethanol (50–100%) for 15-min intervals. After drying, the samples were coated with gold using a Denton Desk-1 Sputter Coater, and examined at an accelerating voltage of 15 KeV using an Amray 1830-D4 SEM equipped with a Tungsten electron gun.

Histological Analysis

Chondrocyte-seeded PLGA scaffolds were fixed with 4% (v/v) phosphate-buffered formaldehyde for 24 h. The samples were then embedded within paraffin wax and cut to 4-μm thickness. The tissue sections were stained with hematoxylin/eosin and safranin-O/fast green.

Immunohistochemical analysis

The section slides (4-μm thick) were rinsed two times in PBS solution, fixed with 4% (v/v) paraformaldehyde for 30 min, rinsed three times in PBS solution again, and then incubated for 15 min in 3% (v/v) H_2O_2. They were rinsed again in tap water for 5 min and incubated for 30 min in 1% (w/v) bovine serum albumin. Specimens were then incubated with the primary antibody overnight at 4°C in a humidified chamber. Immunostaining for collagen types I and II was performed with the corresponded monoclonal

antibodies (Chemicon) that were used at a 1:200 dilution. After rinsing three times with 0.03% (w/v) Triton X-100 in PBS solution, the slides were incubated for 1 h with biotinylated 2nd anti-mouse IgG (Vector), diluted 1:40. Slides were then washed three times with 0.03% Triton X-100 in PBS solution. Specimens were then incubated with avidin peroxidase complex (ABC kit, Vector, Burlingame, CA, U.S.A.) for 30 min. After being washed, slides were incubated for 10 min. Then, the slides were washed in water and mounted with universal mount (Research Genetics).

RESULTS

SEM Analysis

The PLGA microspheres prepared by the oil-in-water emulsion method were basically spherical and the sizes were 40–100 μm in diameter with a smooth surface, as indicated by morphological examination using SEM (Fig. 1A). The surface morphology of the PLGA microspheres incubated in PBS solution for 24 days is shown in Fig. 1B. The degradation of the polymer was started at 0 day after incubation and the microspheres were in the "erosion phase" with the incubation time. Finally, the microspheres were in a broken-down state having the porous surface after 24 days of incubation.

Rabbit articular chondrocytes were seeded on PLGA microspheres and PLGA sponges, and cultured for 5 weeks. After seeding of the cells on the scaffolds, greater numbers of chondrocytes were observed and homogeneously distributed throughout the PLGA microspheres compared with PLGA sponges. This indicated that the PLGA microspheres facilitated cell seeding. At 15 h of culture, morphological difference of the chondrocytes was observed on the PLGA

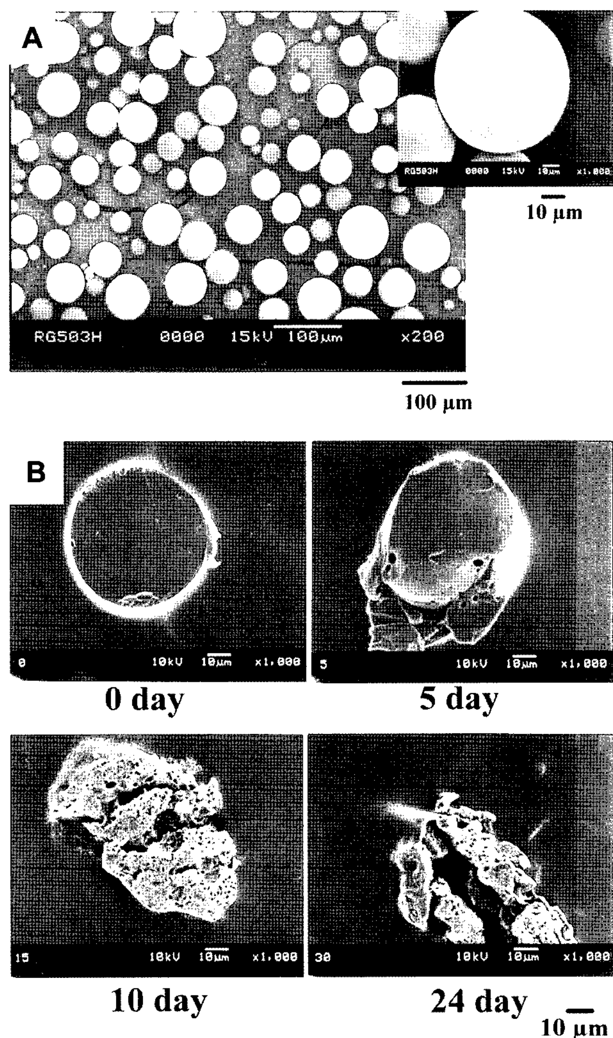

Fig. 1. SEM analysis of PLGA microspheres.
A. The size of microspheres was 40–100 μm in diameter. B. The microspheres were degraded with the incubation time.

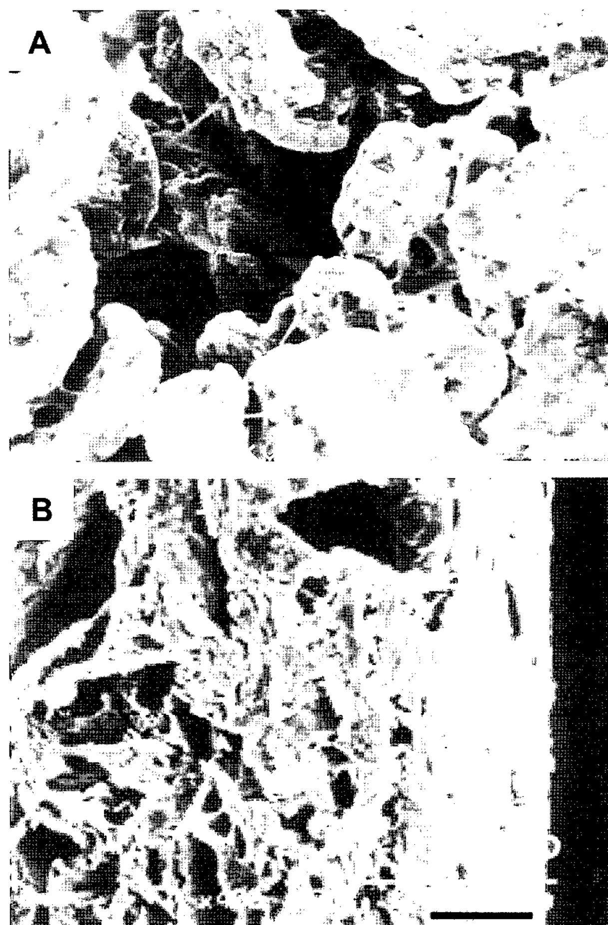

Fig. 2. SEM micrographs of chondrocytes on PLGA microspheres and PLGA sponge scaffolds. Cell attachment on PLGA scaffolds was observed.
A. Chondrocytes were homogeneously distributed throughout the PLGA microspheres. B. Fibroblastic morphologies and accumulation of the chondrocytes were seen on the surface of PLGA sponge (magnification ×400; Bar=100 μm).

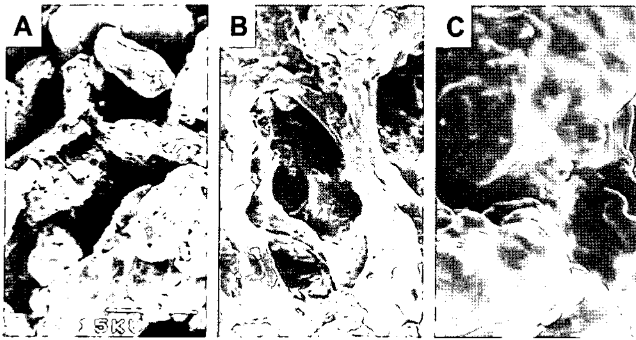

Fig. 3. SEM micrographs of chondrocytes on PLGA microspheres.
The extensive growth of chondrocytes was observed for the cultivation period of 5 weeks. Chondrocyte-seeded PLGA microspheres construct was cultured in culture medium for 15 h (**A**), 4 weeks (**B**), and 5 weeks (**C**) (magnification ×200; Bar=100 μm).

microspheres and PLGA sponge scaffolds. Chondrocytes cultured on PLGA microspheres showed their natural round shapes (Fig. 2A), whereas an the accumulation of fibroblastic chondrocytes was observed on the PLGA sponge scaffold (Fig. 2B). Moreover, the proliferation of chondrocytes on the PLGA microspheres showed with the culture time, synthesizing a large amount of extracellular proteins (Fig. 3). Then, the cells and extracellular proteins

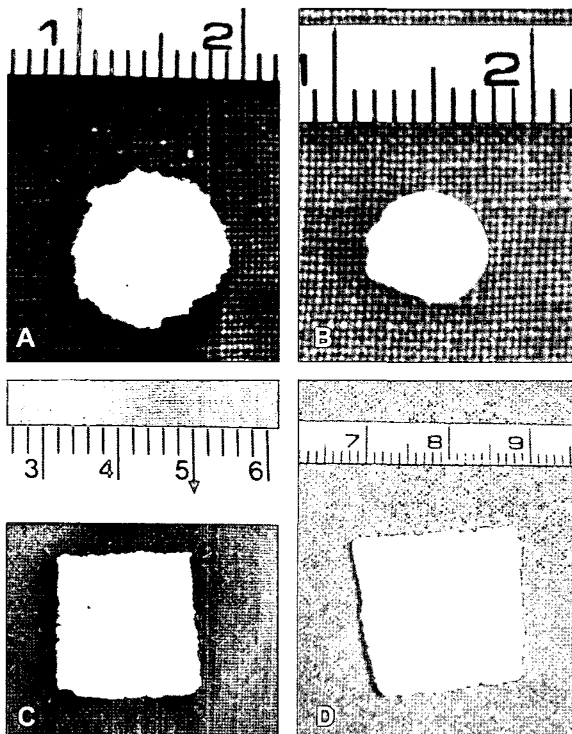

Fig. 4. Fabrication of a cartilaginous tissue by chondrocyte-seeded PLGA microspheres and PLGA sponge.
The construct of chondrocyte-seeded PLGA microspheres was cultured for 2 (**A**) and 4 weeks (**B**). Chondrocyte-seeded PLGA sponge was also cultured for 2 (**C**) and 4 weeks (**D**).

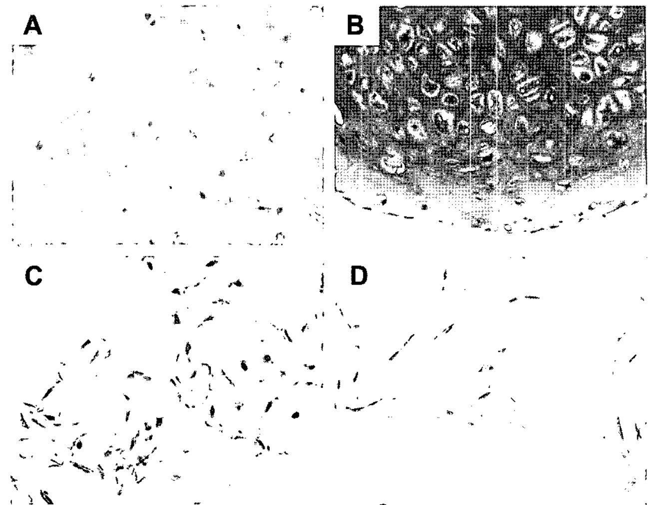

Fig. 5. Hematoxylin/eosin staining of chondrocyte-seeded PLGA scaffolds.
The construct of chondrocyte-seeded PLGA microspheres was cultured for 2 (**A**) and 4 weeks (**B**). Chondrocyte-seeded PLGA sponge was also cultured for 2 (**C**) and 4 weeks (**D**) (magnification ×400).

covered all of the PLGA microspheres (Fig. 3C), finally forming a cartilaginous tissue at 5 weeks of culture.

Cartilaginous Tissue Formation

As shown in Fig. 4, chondrocyte-seeded PLGA microspheres were formed a round construct at 2 weeks of culture (Fig. 4A), which became a cartilaginous tissue at 5 weeks (Fig. 4B). The tissue was similar to an articular cartilage, having a soft texture and whitish color. In the case of chondrocyte-seeded PLGA sponge, any sign of the tissue

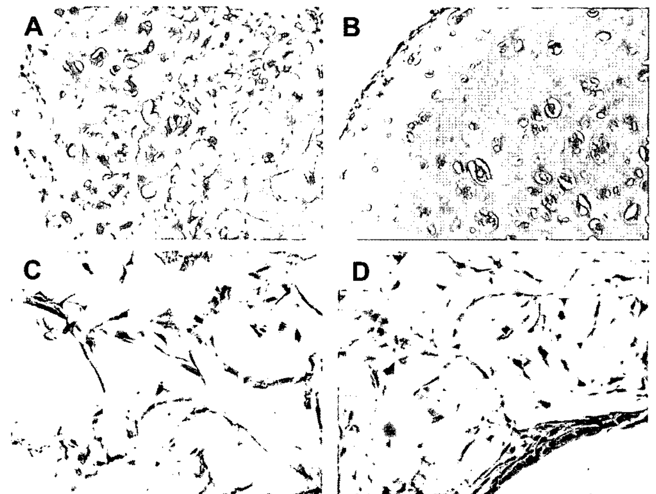

Fig. 6. Safranin O/fast green staining of GAG synthesized by chondrocyte-seeded PLGA scaffolds.
The construct of chondrocyte-seeded PLGA microspheres was cultured for 2 (**A**) and 4 weeks (**B**). Chondrocyte-seeded PLGA sponge was also cultured for 2 (**C**) and 4 weeks (**D**) (magnification ×400).

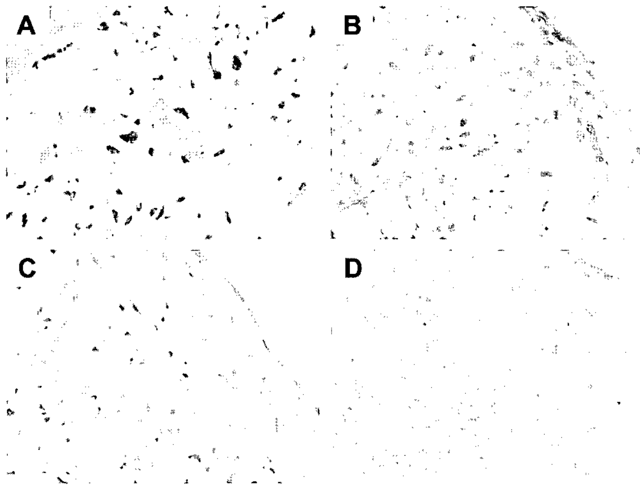

Fig. 7. Immunohistochemical staining of collagen types I and II synthesized by the chondrocyte-seeded PLGA microspheres construct.
Collagen type II staining results after 2 (**A**) and 4 weeks (**B**), and collagen type II staining results after 2 (**C**) and 4 weeks (**D**) are shown on the surface of the PLGA microspheres construct (magnification ×200).

formation was not observed for the culture period of 5 weeks (Figs. 4C and 4D). No significant difference was detected at 1- and 4-week specimens on gross examination.

Histological and Immunohistochemical Examination

Histological analysis of the chondrocyte-seeded PLGA microspheres construct using hematoxylin/eosin stains showed the increase of round morphological chondrocytes in lacunae, separated from the interterritorial extracellular matrix with the culture time (Figs. 5A and 5B), whereas the morphologies of the cells in the PLGA sponge were changed to fibroblastoid after 5 weeks (Figs. 5C and 5D). Safranin O/fast green staining demonstrated that cartilaginous extracelluar matrix produced by chondrocytes on the PLGA microspheres construct consisted mainly of glycosaminoglycans and increased with the culture time (Figs. 6A and 6B). Safranin-O was homogeneously bound to almost the entire cross-section of the engineered tissue construct after 5 weeks (Fig. 6B), whereas the production of glycosaminoglycans was not oberserved in the PLGA sponge (Figs. 6C and 6D). The presence of collagen type II was detected in the construct of PLGA microspheres using the immunohistochemical staining method, after 2 and 5 weeks of culture (Figs. 7A and 7B). The production of collagen type I, however, was not observed (Figs. 7C and 7D).

DISCUSSION

The scaffold should be porous and permeable to allow for the ingress of nutrients and removal of waste products, while providing a temporary physical support for cells and extracellular matrix components. Generally high porosity and strong mechanical support can be contradictory to each other. A more porous scaffold is likely to be weaker. The microsphere system completely ignored the mechanical support, and instead induced the extracellular matrix to aggregate one another, finally forming a mass.

The PLGA microspheres provided enough surface area to attach the cells that were completely exposed to media, resulting in no mass transfer limitation. Furthermore, the PLGA microspheres provided a good microenvironment for cell-cell and cell-matrix interaction, which improved cell proliferation and production of cartilaginous matrix such as proteoglycan and collagen type II without change of their phenotypical round morphology for 5 weeks of culture. Moreover, the degradation of PLGA microspheres, as shown in Fig. 1B, was likely to promote the formation of homogenous cartilage tissue during synthesizing of cartilaginous proteins by the adhesive cells, finally fabricating a cartilaginous tissue.

Frondoza *et al.* [10] evaluated 4 kinds of microcarriers to develop a human articular chondrocytes expansion system, maintaining their cellular phenotype. They found that collagen type I beads, 100–400 μm in diameter, promoted cell proliferation up to 20 times and enhanced re-expression of cartilaginous extracellular proteins. The cells attached on the surface of microcarriers appeared spherical, and this alteration of cell morphology seemed to promote the production of cartilaginous proteins.

Another stimulating factor for the chondrogenic effect was that the cells were exposed to mechanical stirring. Mechanical load is very essential for chondrocytes to grow, which are very anchorage- and stress-dependent cells [1, 5]. The free fluid by the stirring process through the cultivation on the rocking platform must have generated various stresses and delivered them to the cells, which in turn encouraged production of extracellular matrix. The size of the PLGA microspheres used in this study was about 40–100 mm in diameter, and chondrocytes seeded on the PLGA microspheres were exposed to stirring force during the cultivation time. Moreover, the interconnected structure between the cell and PLGA microspheres resulted in a spatial uniform cell distribution throughout the PLGA microspheres. These factors are likely to make the microspheres a more cartilaginous construct, and therefore, the PLGA microspheres promote the homogeneous distribution of chondrocytes and synthesis of cartilaginous matrix.

To the best of our knowledge, this is the first approach to fabricating cartilage *in vitro* with a powder form scaffold. It can be applied to other synthetic and natural polymers as well. The optimal seeding density of cells for powder polymers and the mechanical properties of the polymers should be investigated to fabricate a more natural cartilage. Application of optimal mechanical stimulation to the cells must also play a crucial role.

In conclusion, PLGA microspheres are valuable to be used as a scaffold for cartilage tissue engineering. Further, *in vivo* studies such as transplantation to immune-competent animals are required to evaluate the quality of the engineered cartilaginous tissue for future clinical use in repair of articular cartilage defects.

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