Macromolecular Research, Vol. 13, No. 4, pp 285-292 (2005)

Chondrogenic Differentiation of Bone Marrow Stromal Cells in Transforming Growth Factor-β_1 Loaded Alginate Bead

Ki Suk Park, Chae Moon Jin, Soon Hee Kim, John M. Rhee, and Gilson Khang*

Department of Polymer·Nano Science and Technology, Chonbuk National University, Jeonju 561-756, Korea

Chang Whan Han

Department of Orthopedic Surgery, College of Medicine, The Catholic University of Korea, Daejeon 301-723, Korea

Yoon Sun Yang

Medipost Co., West Bldg., 3rd floor, Doosan Technical Institute, 39-3, Seongbok, Yongin, Gyeonggi 449-140, Korea

Moon Suk Kim and Hai Bang Lee

Nanobiomaterials Laboratory, Korea Research Institute of Chemical Technology, P.O.Box 107, Yusung, Daejeon 305-600, Korea

Received January 11, 2005; Revised June 29, 2005

Abstract: We developed alginate beads loaded with transforming growth factor-β_1 (TGF-β_1) to examine the possible application of the scaffold and cytokine carrier in tissue engineering. In this study, bone marrow stromal cells (BMSCs) and TGF-β_1 were uniformly encapsulated in the alginate beads and then cultured *in vitro*. The cell morphology and shape of the alginate beads were observed using inverted microscope, scanning electron microscope (SEM), histological staining and RT-PCR to confirm chondrogenic differentiation. The amount of the TGF-β_1 released from the TGF-β_1 loaded alginate beads was analyzed for 28 days *in vitro* in a phosphate buffered saline (pH 7.4) at 37 °C. We observed the release profile of TGF-β_1 from TGF-β_1 loaded alginate beads with a sustained release pattern for 35 days. Microscopic observation showed the open cell pore structure and abundant cells with a round morphology in the alginate beads. In addition, histology and RT-PCR results revealed the evidence of chondrogenic differentiation in the beads. In conclusion, these results confirmed that TGF-β_1 loaded alginate beads provide excellent conditions for chondrogenic differentiation.

Keywords: TGF-β_1, alginate bead culture, chondrogenic differentiation, stromal cell, controlled release.

Introduction

Articular cartilage has a very poor capacity for spontaneous healing since the avascular environment in this tissue is poor. Over the past few decades, several studies have been conducted regarding repair techniques for defected cartilage treatment. Recent studies have focused on the cell-based repair as tissue engineering for defected cartilage treatment.

Tissue engineering generally combines 3 key elements; matrices (scaffolds), signaling molecules (growth factors), and cells. Recently, tissue engineering has been introduced to develop cell-based repair of defected organ and tissue.[1-15] These theoretical concepts are a basis for artificial matrices, which are a scaffold of cell proliferation and regeneration. Growth factors are known as promoters of the cell and tissue growth and inducers for reconstruction with other elements. Therefore, these factors are considered as essential in tissue engineering.[16-18] Growth factors, a type of water-soluble polypeptides are synthesized and secreted from cells in various tissues, and generally acts on nearby cells. They have a specific structure effecting cell bioactivity and proliferation of specific cells.[19-23] Almost of all growth factors have a very short half-life and are toxic in high dosage; therefore, the stabilization of growth factors in delivery devices and the design of delivery carriers are important factors for growth factor delivery, in order to achieve a controlled release.[5]

The ideal growth factor carrier for transplantation *in vivo* should be one that most closely mimics the natural *in vivo* environment. Alginate, a natural biopolymer extracted from brown algae, has several unique properties as a family of

*Corresponding Author. E-mail: gskhang@chonbuk.ac.kr

linear unbranched polysaccharides that contain varying amounts of 1,4′-linked β-D-mannuronic acid and α-L-guluronic acid residues.[24] It was widely used to produce microspheres, beads, microcapsules, and tablets for drug delivery systems, and a matrix for tissue engineering.[25] Alginate can be ionically crosslinked by the addition of divalent cations such as Ca^{2+} and Ba^{2+} in aqueous solution. An ionical gelation process has enabled not proteins, but cells and DNA to be incorporated into alginate matrices with retention of full biological activity. Moreover, the pore size, degradation rate, and ultimately release kinetics, possible to control by selecting the type of alginate and additive.[26, 27]

Over the past few years, a considerable number of studies have been conducted on cartilage regeneration by cells encapsulated in alginate bead, but very few attempts have been made at cartilage regeneration by cells encapsulated in a growth factor loaded alginate bead. We observed the release profile of the TGF-β_1 from TGF-β_1 loaded alginate bead with a zero-order release pattern for 35 days and concluded that the alginate bead could possibly used as cell scaffold.[28] In this study, we encapsulated BMSCs into TGF-β_1 loaded alginate bead with $CaCl_2$ as cross-linking agent and added heparin to improve the biocompatibility of sodium alginate and stabilize TGF-β_1. Structure and histological analysis were observed by SEM, hematoxylin and eosin (H&E) staining and Safranin-O staining. In addition, RT-PCR performed to identify the type αI (II) collagen gene existing in the alginate bead.

Experimental

Materials. Tissue culture reagents Dulbecco's modified Eagle's medium (DMEM), antibiotic-antimycotic solution, phosphate buffered saline (PBS), trypsin-EDTA, and fetal bovine serum (FBS) and 100 mM dNTP set (dATP, dCTP, dGTP, dTTP) were purchased from Gibco BRL® (Grand Island, NY, USA). Low viscosity (250 cps in 2% solution at 25 °C) sodium alginate, calcium chloride ($CaCl_2$) and glutaraldehyde purchased from Sigma Chemical Co. (St. Louis, MO, USA). ELISA kit (Quantikine™ human TGF-β_1) and rhTGF-β_1 were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Cell culture wares purchased from Falcon® (Becton & Dickinson Labware, USA). Heparin purchased from Choongwae Corp. (Korea). RT-PCR reagents (TriZol®, RNase inhibitor, SuperScript™ II RNase H$^-$ reverse transcriptase set, oligo $dT_{12\text{-}18}$ primer, and DNA ladder) and PCR master were purchased from Invitrogen™ (California, USA) and Roche Molecular Biochemicals (Mannheim, Germany). Primer synthesized in Geno Tech Corp. (Korea). All other chemicals were used cell culture tested grades.

Cell Isolation and Culture. Bone marrows donated from human donors at the St. Mary's hospital in Daejeon. Briefly, 25 mL of heparinized bone marrow was mixed with an equal volume of high glucose-DMEM medium, supplemented with an antibiotic-antimycotic solution (100 U/mL penicillin G, 100 μg/mL streptomycin, 250 ng/mL amphotericin B) and 10% FBS. For stromal cells isolation, marrow was layered onto a percoll cushion and centrifuged for 25 min at 2,500 rpm. The nucleated cell fraction was plated in tissue-cultured flasks and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. When these primary BMSCs reached 80~90% of confluence, they were trypsinized, and passaged 4 or 5 times. The medium was changed every 2-3 days.

Preparation and Culture of Alginate Beads. After 4^{th}~5^{th} passage, the BMSCs were encapsulated in an alginate bead. Low viscosity sodium alginate sterilized by UV illumination overnight, and then sterilized sodium alginate dissolved at 1.2% (w/v) in a filtrated PBS. Then, TGF-β_1 mixed in an alginate solution to make two groups with a final concentration of 0.5 and 1.0 μg/mL, respectively. Each group was composed of two types with/without 0.1 mg/mL heparin. The cell was trypsinized and resuspended in four types of alginate solution. The cell suspension was gently mixed and then dropped through a 23-gauge syringe needle into 102 mM $CaCl_2$ solution. The bead dropped in a $CaCl_2$ solution for 10 min to allow full crosslinking reaction. Then the bead washed three times, twice with PBS and last with a complete medium. The medium changed every 2-3 days.

SEM. The influence of material property on cell morphology was conducted using SEM. After 14 days of incubation, the bead was rinsed three times with PBS and fixed overnight at 4 °C in 2.5% glutaraldehyde in PBS. The specimen was then dehydrated in a graded series of ethanol and dried by air. Before analysis, the specimen was mounted onto aluminum stubs and sputtered with gold. The specimen was examined and photographed using S-2250N SEM (Hitachi, Japan) at 15 kV.

Determination of the Release Profile. 0.5 and 1.0 μg of TGF-β_1 loaded alginate beads were immersed in 10 mL of PBS and incubated in a 5% CO_2 contained air at 37 °C. At a scheduled time, the supernatant 1 mL of alginate beads was collected and replaced with the same volume of fresh PBS, followed by gently mixing. The aliquots were stored at -20 °C prior to analysis. The concentration of TGF-β_1 released from alginate beads over time was measured by the TGF-β_1-ELISA in comparison to a calibration curve. The color development was stopped and the intensity of the color was measured; absorbance was measured at 450 nm using an ELISA plate reader (E-max, Molecular Device Co., USA).

Histochemical Staining. Antibody staining was carried out to determine histological morphology. Alginate beads were fixed in 10% formalin in PBS for 2 hrs at room temperature, after being washed with PBS. Then, the beads were dehydrated through a graded series of ethanol to absolute ethanol before embedding them in paraffin and cutting 5 μm sections using a LEICA RM2135 (Germany).

H&E staining was used to observe the distribution of cells that had encapsulated in the alginate gel. Sulfated glyco-

saminoglycans were stained using Safranin-O staining. The stained slide was counterstained with Mayer's hematoxylin. The slide was viewed and photographed using an inverted microscope (Nikon Diapot, Japan).

RNA Isolation and RT-PCR. Total RNA was extracted from BMSCs cultured on the alginate bead using a TriZol® reagent, as recommended by the manufacturer. The total RNA was used for cDNA synthesis using an oligo dT_{12-18} primer and SuperScript™ II RNase H⁻ reverse transcriptase. RT-PCR analysis was performed for the human genes type α1 (II) collagen and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) using the primer sequences provided (Table I). The housekeeping gene GAPDH was used as a control. In summary, 5 μL of single stranded cDNA was used to seed a 50 μL PCR, which was subjected to cycles 24 and 25. Following an initial denaturation for 4 min at 95 °C, a typical amplification cycle consisted of 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min, ending with a 10 min extension at 72 °C (GAPDH) and 95 °C for 3 min, 58 °C for 2 min and 72 °C for 1 min 30 s ending with a 10 min extension at 72 °C, which was performed using a Gene cycler™ thermal cycler (BIO-RAD, CA, USA). Following the designated number of cycles, 8 μL of the reaction was removed and the product was separated and visualized in a 2% agarose gel containing 1 μg/mL ethidium bromide after which gels were viewed using an UV transilluminator (Spectroline®, NY, USA) and photographed using a Gel Cam (Polaroid, UK).

Results and Discussion

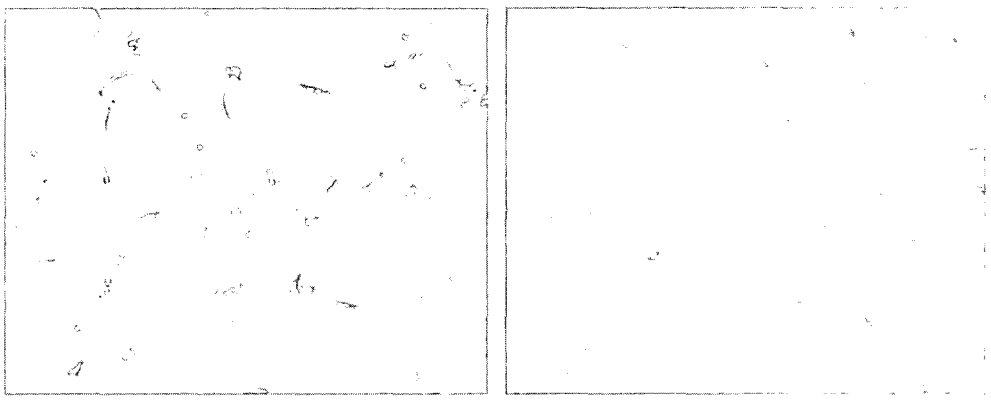
Cell Expansion. BMSCs were isolated from human bone marrow cultured in monolayer in the presence of 10% fetal bovine serum, and observed fibroblast-like morphology (Figure 1). Adult BMSCs were expanded as undifferentiated cells in culture for more than 15 passages, an indication of their proliferate capacity.[14,15] No differences were found around the passages.

Morphology of Alginate Beads. TGF-β_1 loaded alginate solution made about 100 beads per 1 mL and the average diameter of the beads was about 2.5 mm. Also, the beads maintained a sphere-like shape for 5 weeks *in vitro* at pH 7.4 and 37 °C (Figures 2 and 3(a)). Microscopic observation of the alginate beads provided evidence of an interconnected network of channels (Figure 3(b)). It appears that the interconnected network of channels acted to provide effective conditions for nearby cells adhesion, movement, and proliferation, that is to say, a tissue engineered scaffold. The morphology of BMSCs in alginate beads were observed by microscope (Figure 2(a), arrow). SEM observation revealed abundant adhesive cells on the channel of alginate bead (Figure 3(c) and (d), arrow).

TGF-β_1 Release Profiles from Alginate Beads. In hydrogels, drugs contact with water and thus the drug solubility is an important factor in drug release. The release of drugs with appreciable water solubility will be rapid and independent of the matrix degradation rate. Thus, in general, hydrogels may not be suitable for controlled release of

Table I. The Sequence of PCR Primer. GAPDH is House Keeping Gene and Type α1 (II) Collagen is Specific Gene of Cartilage

Primer	Sequence	Size
GAPDH	sense: 5'-ACCACAGTCCATGCCATCAC-3' antisense: 5'-TCCACCACCCTGTTGCTGTA-3'	450bp
Type α1 (II) collagen	sense: 5'-CTGCTCGTCGCCGCTGTCCTT-3' antisense: 5'-AAGGGTCCCAGGTTCTCCATC-3'	359bp

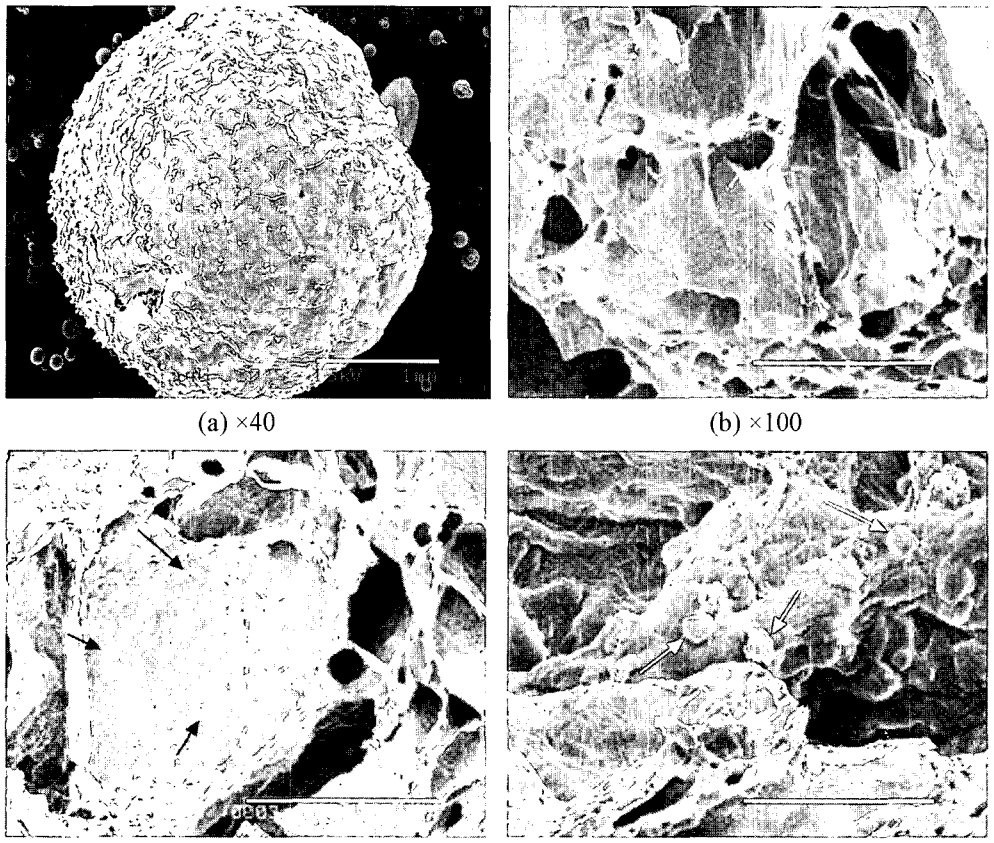


(a) (b)

Figure 1. Proliferation of BMSCs. BMSCs culture was maintained in a proliferation medium. (a) is primary culture for 5 days and (b) is culture for 10 days (× 40).

(a) ×60 (b) ×60

Figure 2. BMSCs were encapsulated in alginate beads. Mixed alginate solution and cell suspension were dropped through a 23-gauze needle into 102 mM $CaCl_2$ to form microsphere beads. (a) is an alginate bead without BMSCs and (b) is an alginate bead encapsulated in BMSCs with TGF-β_1 (arrow is BMSCs encapsulated in the bead).



(a) ×40 (b) ×100

(c) ×100 (d) ×1000

Figure 3. The morphology of an alginate bead observed by SEM. (a) and (b) is an alginate bead without BMSCs, and (c) and (d) is an alginate bead encapsulated BMSCs with TGF-β_1. Black and white arrow (c and d) directed adhesive cells on the channel of alginate bead (scale bar: (a) 1 mm, (b) and (c) 500 μm, (d) 50 μm).

low molecular weight and water-soluble drugs. The hydrogel systems, however, are useful for the delivery of macromolecular drugs, such as peptides and proteins, which are entrapped in the gel network until the gel is degraded. We prepared alginate beads containing TGF-β_1. In fact, a two-phase release profile of TGF-β_1 from the different batches

of alginate beads monitored by ELISA for the sample PBS solution was observed as shown in Figure 4. In the first phase (0~7 days), release was increased up to about 64.29 pg/day. The second phase (7~35 days) of TGF-β_1 release became blunt to about 12.5 pg/day. These results were explained because of calcium-alginate disintegration behavior. Protein loaded alginate matrices are release by two mechanisms. One is diffusion of the protein through the pores of the polymer network. The other is degradation of the polymer network. Calcium-alginate gel disintegrates through the ion exchange of calcium ions chelated with the carboxylate anions in the alginate and sodium and potassium ions in the PBS.[19] In this research, initial burst (0~7 days) induced diffusion from TGF-β_1 existed on surface of alginate beads. Therefore, initial release profile revealed a relative large quantity of TGF-β_1. Then the release of TGF-β_1 in the second phase resulted form polymer network disintegration of calcium-alginate beads by Ca-K or Ca-Na ion exchange reaction. However, the beads maintained a globular shape above 30 days. We attributed this result to decrease of potassium and sodium ion in PBS and this phenomenon caused slower release profile in second phase than initial phase. The addition of heparin in alginate beads did not reduce the burst effect compared with alginate beads without heparin. But the addition of heparin in alginate beads reduced the release amount and percentage compared with alginate beads without heparin after 5 days (Figure 4). This phenomenon caused that heparin, macromolecular proteins, interrupted TGF-β_1 release by difference of concentration of inside and outside gel. Neither batch showed a different release pattern according to the loading amount of TGF-β_1 in alginate beads. From this result, we could confirm to release only 0.3% of TGF-β_1 for 35 days. However, we showed a similar tendency in deferent research, and current studies for this release tendency in alginate beads.[29-31]

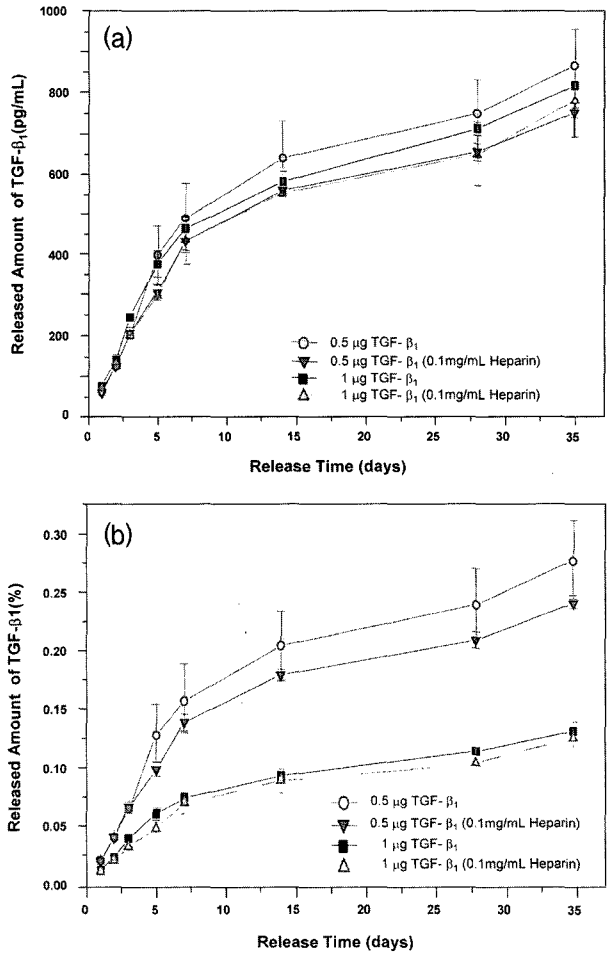


Figure 4. Release profile of TGF-β_1 from alginate beads. (a) is release amount of TGF-β_1 expressed by pg/mL, and (b) is release amount of TGF-β_1 expressed by percentage.

Histology. After 14 days of incubation, gross inspection revealed semitransparent white masses embedded in BMSCs encapsulating alginate bead surface. H&E stained sections revealed evidence of cartilage-like tissue, such as lacuna space within alginate beads (Figure 5). Histologically, these correlated with clusters of cells surrounded by Safranin-O positive extracellular matrix (Figure 6). Furthermore, we did not observe a change of GAG expression by TGF-β_1 amount, or heparin addition. The alginate bead without cells did not observe GAG expression, or any tissue-like shapes (Figure 7).

RT-PCR Analysis. RT-PCR analysis further confirmed the chondrogenic differentiation in the TGF-β_1 loaded alginate bead. In order to determine the expression of extracellular matrix components during differentiation in TGF-β_1, loaded alginate bead were cultured for up to 14 days and probed by RT-PCR. Only TGF-β_1 loaded beads expressed type α1 (II) collagen. This cartilage-specific transcript was detected at 14 days (Figure 8). This qualitative result did not reveal striking differences between the TGF-β_1 dose-dependant. However, these findings are noteworthy. This finding indicated that, although these marrow-derived cells were multipotential, they preferentially differentiated into the chondrogenic phenotype within the three-dimensional constructs when loaded with TGF-β_1.

Conclusions

Microscopic observation showed the open cell pore structure and abundant cells with a round morphology in alginate beads. Histology and RT-PCR results revealed evidence of chondrogenic differentiation in the beads. Depending on its composition, TGF-β_1 loaded alginate gel can act as a substrate for BMSCs proliferation, and has potential for use as 3D degradable scaffolds. Alginate beads incorporated with TGF-β_1 prolonged TGF-β_1 release from alginate beads, and stabilized TGF-β_1 during long period of release. From the result of an *in vitro* release test, no batch exhibited different

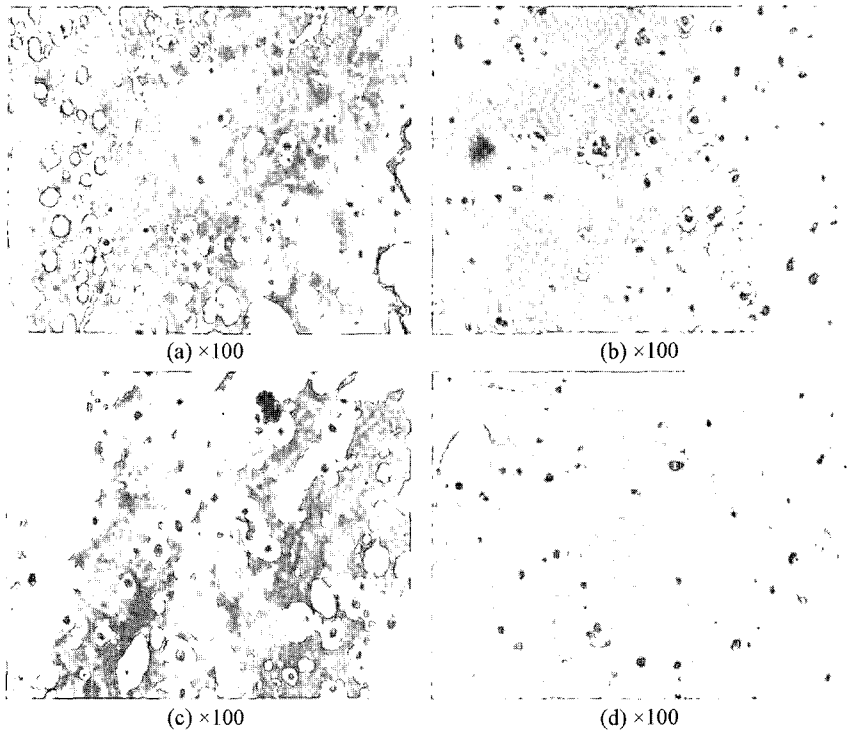


Figure 5. Differentiated BMSCs showed a lacuna, in which we stained the nucleus by H&E, were surrounded by sulfated glycosaminoglycan secreted in an extracellular matrix. (a) 0.5 μg/mL TGF-β_1, (b) 1.0 μg/mL TGF-β_1, (c) 0.5 μg/mL TGF-β_1 with heparin, and (d) 1.0 μg/mL TGF-β_1 with heparin.

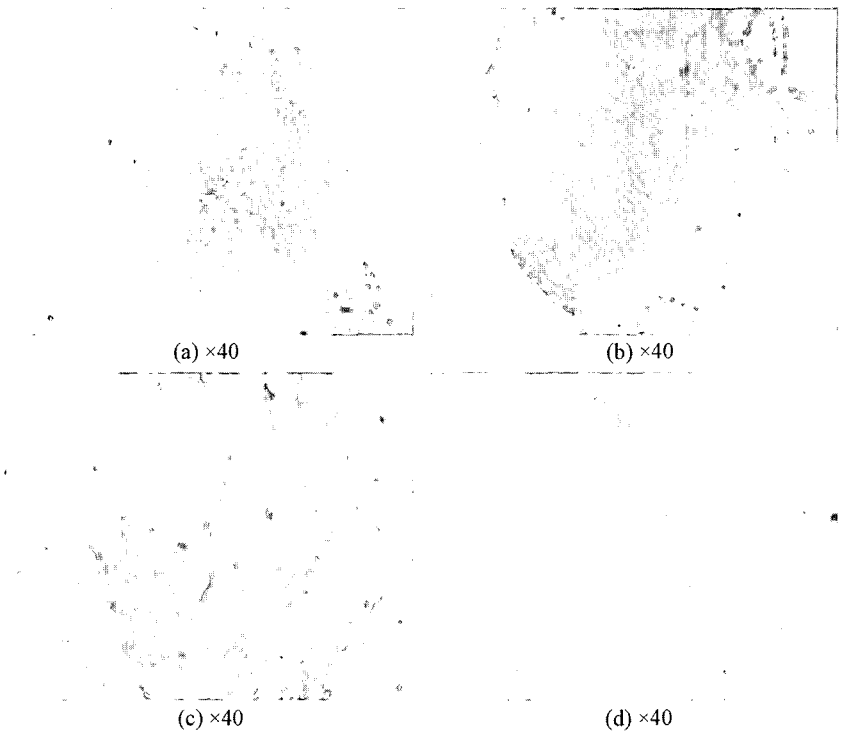
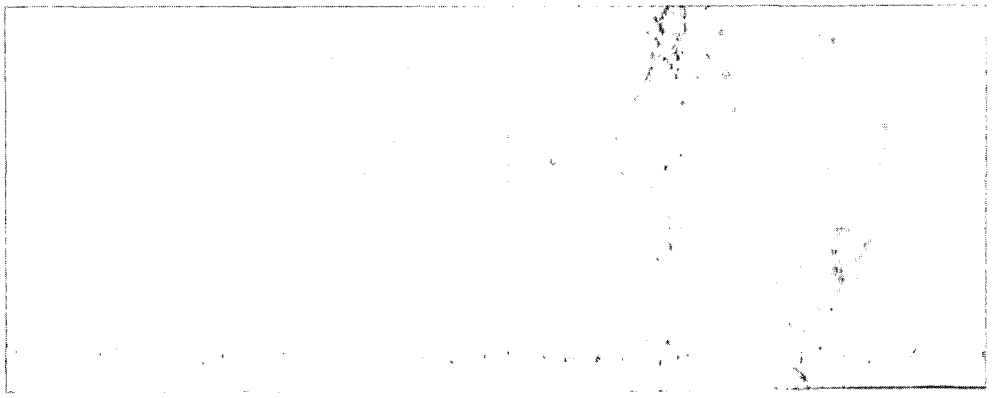


Figure 6. After 2 weeks in culture, Safranin-O staining for sulfated glycosaminoglycan to be evaluated chondrogenic differentiation showed a progressive increase in Safranin-O staining intensity, compared with alginate beads without cells. (a) 0.5 μg/mL TGF-β_1, (b) 1.0 μg/mL TGF-β_1, (c) 0.5 μg/mL TGF-β_1 with heparin, and (d) 1.0 μg/mL TGF-β_1 with heparin.



(a) ×40 (b) ×100

Figure 7. After 2 weeks in culture, Safranin-O staining for sulfated glycosaminoglycan to be evaluated chondrogenic differentiation showed that an alginate bead without BMSCs stained weakly as well as did not show cells in a lacuna.

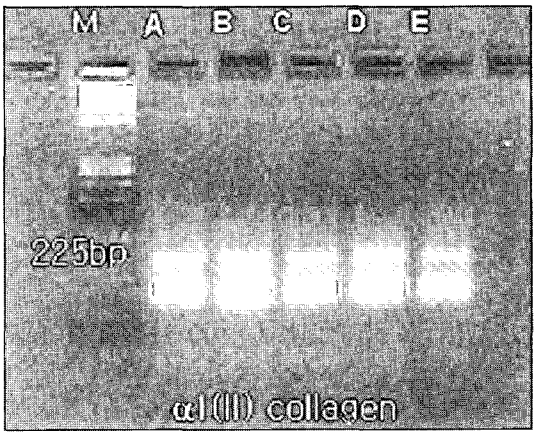


Figure 8. After RNA isolated from cultured BMSCs on alginate bead culture for 14 days, RT-PCR was carried out for type α1 (II) collagen expression of differentiated BMSCs. Type α1 (II) collagen which is a specific gene of cartilage expressed each dose-dependant loading TGF-β_1 in the alginate bead. A. 0.5 μg/mL TGF-β_1, B. 1.0 μg/mL TGF-β_1, C. 0.5 μg/mL TGF-β_1 with heparin, D. 1.0 μg/mL TGF-β_1 with heparin and E. no loading TGF-β_1.

release patterns following a loading amount of TGF-β_1 in alginate beads, and observed slow released patterns. The release profile of TGF-β_1 was observed for two separated release patterns. These results were explained because of calcium-alginate disintegration behavior as mentioned above.[23]

In conclusion, these results proved that TGF-β_1 is an important factor in chondrogenic differentiation and TGF-β_1 loaded alginate beads provided excellent conditions for cell encapsulation into alginate bead for chondrogenic differentiation. Studies regarding more long-term *in vitro* experiments proposed this system, *in vivo* animal experiment for chondrogenic differentiation in TGF-β_1 controlled released alginate beads and the relation with osteogenic and chondrogenic differentiation in alginate beads with BMSCs are in progress.

Acknowledgements. This work was supported by KMOHW (0405-BO01-0204-0006) and SCRC (SC3100).

References

(1) G. Khang, S. J. Lee, M. S. Kim, and H. B. Lee, in *Webster's Biomedical Engineering Handbook*, S. Webster, Ed., John & Wiley Press, NY, *in press*, 2005.
(2) G. Khang, J. H. Lee, and H. B. Lee, in *CRC Biomedical Engineering Handbook*, 2nd Ed., J. Bronzio, Ed., CRC Press, Boca Raton, FL, 2000, Section IV, Chap. 39, pp 1-23.
(3) G. Khang and H. B. Lee, in *Methods of Tissue Engineering*, A. Atala and R. Lanza, Eds., Academic Press, 2001, Section II, Chap. 20, pp 771-780.
(4) G. Khang and H. B. Lee, *Biomedical Polymers*, Korean Chemical Society Press, Munundang, Seoul, Korea, 2001.
(5) E. K. Jeon, H. J. Whang, G. Khang, I. Lee, J. M. Rhee, and H. B. Lee, *Polymer(Korea)*, **25**, 893 (2001).
(6) E. K. Jeon, J. Y. Shim, H. J. Whang, G. Khang, I. Jo, I. Lee, J. M. Rhee, and H. B. Lee, *Biomater. Res.*, **5**, 23 (2001).
(7) J. W. Jang, B. Lee, C. W. Han, I. Lee, H. B. Lee, and G. Khang, *Polymer(Korea)*, **27**, 226 (2003).
(8) G. Khang, E. K. Jeon, J. M. Rhee, I. Lee, S. J. Lee, and H. B. Lee, *Macromol. Res.*, **11**, 334 (2003).
(9) H. B. Lee, G. Khang, and S. J. Lee, in *Tissue Engineering and Regenerative Medicine*, J. J. Yoo and I. Lee, Eds., Gunja Pub., Seoul, 2002, Chap. 41, pp 751-770.
(10) G. Khang, S. J. Lee, J. M. Rhee, and H. B. Lee, in *Tissue Engineering and Regenerative Medicine*, J. J. Yoo and I. Lee, Eds., Gunja Pub., Seoul, 2002, Chap. 17, pp 297-322.
(11) S. J. Lee, I. Lee, Y. M. Lee, H. B. Lee, and G. Khang, *J. Biomater. Sci. Polym. Ed.*, **15**, 1003 (2004).
(12) J. W. Jang, B. Lee, C. W. Han, M. S. Kim, S. H. Cho, H. B. Lee, and G. Khang, *Polymer(Korea)*, **28**, 382 (2004).
(13) H. J. Hwang, G. Khang, J. H. Sung, I. Lee, and H. B. Lee, *Biomater. Res.*, **6**, 45 (2002).
(14) G. Khang, P. K. Shin, I. Y. Kim, B. Lee, S. J. Lee, Y. M. Lee,

H. B. Lee, and I. Lee, *Macromol. Res.*, **10**, 158 (2002).
(15) M. F. Pittenger, A. M. Mackay, S. C. Beck, and S. Craig, *Science*, **284**, 143 (1999).
(16) S. P. Baldwin and W. M. Saltzman, *Adv. Drug Del. Rev.*, **33**, 71 (1998).
(17) Y. Tabata, *PSTT*, **3**, 80 (2000).
(18) R. Langer, *J. Control. Rel.*, **62**, 7 (1999).
(19) T. A. Holland and A. G. Mikos, *J. Control. Rel.*, **86**, 1 (2003).
(20) S. Kondo, S. H. Cha, W. F. Xie, and L. J. Sandell, *J. Ortho. Res.*, **19**, 712 (2001).
(21) H. Mizuta, A. Sanyal, T. Fukumoto, J. S. Fitzsimmons, N. Matsui, M. E. Bolander, M. J. Oursler, and S. W. O'Driscoll, *J. Ortho. Res.*, **20**, 565 (2002).
(22) J. W. Lee, W. N. Qi, and S. P. Scully, *J. Ortho. Res.*, **20**, 66 (2002).
(23) A. Vila, A. Sánchez, M. Tobío, P. Calvo, and M. J. Alonso, *J. Control. Rel.*, **78**, 15 (2002).
(24) W. R. Gombotz and S. F. Wee, *Adv. Drug Del. Rev.*, **31**, 267 (1998).
(25) R. Huang, Y. Du, and J. Yang, *Carbo. Polymer*, **52**, 19 (2003).
(26) J. H. Kim, J. M. Oh, G. Khang, J. K. Jeong, J. S. Lee, S. Y. Jeung, and H. B. Lee, *J. Kor. Pharm. Sci.*, **32**, 277 (2002).
(27) M. Weber, A. Steinert, A. Jork, A. Dimmler, F. Thürmer, N. Schütze, C. Hendrich, and U. Zimmermann, *Biomaterials*, **23**, 2003 (2002).
(28) K. S. Park, E. J. Kim, M. S. Kim, S. H. Cho, J. M. Rhee, H. B. Lee, and G. Khang, *Biomater. Res.*, **8**, 14 (2004).
(29) M. Mierisch, B. Cohen, C. Jordan, and G. Robertson, *J. Arthro. Rel.*, **18**, 892 (2002).
(30) R. J. Mumper, A. S. Huffman, P. A. Puolakkainen, L. S. Bouchard, and W. R. Gombotz, *J. Control. Rel.*, **30**, 241 (1994).
(31) G. W. Vandenberg, C. Drolet, S. L. Scott, and J. de la Noue, *J. Control. Rel.*, **77**, 297 (2001).
(32) K. Fu, A. M. Klibanov, and R. Langer, *Nature Biotech.*, **18**, 24 (2000).
(33) A. Kikuchi, M. Kawabuchi, M. Sugihara, Y. Sakurai, and T. Okano, *J. Control. Rel.*, **47**, 21 (1997).