Chondrogenesis of myoblasts in biodegradable poly-lactide-co-glycolide scaffolds

YANGLIN GU[1], PENG CHEN[1], YUSHENG YANG[1], KEQIN SHI[1], YUBIN WANG[2], WENHUI ZHU[2] and GUOXING ZHU[1]

[1]Department of Orthopedics, Wuxi No. 2 People's Hospital, Jiangsu 214002; [2]Department of Sports Medicine, Dongfang Hospital Affiliated to Tongji University, Shanghai 200120, P.R. China

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Abstract. Myoblasts are considered to be an alternative cell source for cell-based meniscal repair due to their multiple differentiation potentials. This study addresses the chondrogenic differentiation of myoblasts seeded into poly-lactide-co-glycolide (PLGA) scaffolds following implantation in a subcutaneous pocket of nude mice. Canine myoblasts isolated from a Beagle were expanded and seeded into PLGA scaffolds and cultured in cartilage-derived morphogenetic protein-2 (CDMP-2) and transforming growth factor-β1 (TGF-β1)-containing medium for 2 weeks *in vitro*. The constructs were implanted into a subcutaneous pocket of 24 combined immunodeficiency mice and harvested after 8 and 12 weeks, respectively. Hematoxylin and eosin staining of the sections of the engineered cartilage at 8 and 12 weeks revealed the regeneration of fibrocartilage. Immunohistochemical staining confirmed a similar distribution of collagen type II in the engineered cartilage as the normal meniscus. At 12 weeks, expression of mRNAs for type I collagen, type II collagen and aggrecan was detected by RT-PCR. The compressive moduli of engineered cartilage reached 85.72% of the normal meniscus at 12 weeks, with a high level of glycosaminoglycan (GAG) content (no statistical difference from normal). Myoblast-seeded PLGA scaffolds express a stable chondrogenic phenotype in a heterotopic model of cartilage transplantation and represent a suitable tool for tissue engineering of cartilage.

Introduction

Meniscus has limited potential of self-repair, and menisci injury may lead to long-term degenerative joint changes (1,2). In addition to other therapies, meniscal regeneration using tissue engineering techniques has been attempted, based on the loading and culture of suitable cells into appropriate scaffolds (3,4). With regards to the cell source, meniscal chondrocytes, mesenchymal cells and pluripotential fibroblasts have all been identified as possible sources for the repair of meniscal tissue (5-7). Compared with the cell sources mentioned above, myoblasts are a promising source for meniscal engineering, as they are relatively abundant and easily accessible with minimal donor site morbidity. Myoblasts may also be good candidates for tissue engineering as they have a higher cell yield and proliferate rapidly during *in vitro* expansion (8).

With regards to the scaffold, a variety of natural or artificial biomaterials have been investigated for use in tissue-engineered meniscus. Due to the poor biomechanical properties and the rapid degradation of fibrin and alginate, polymer scaffolds with a stable, biodegradable and permeable pore network were used to support cell attachment, proliferation and nutrient exchange and to provide stability (5,9).

In this study, non-woven PLGA scaffolds were seeded with myoblasts and transplanted into subcutaneous pockets of 24 combined immunodeficiency (SCID) mice. It was hypothesized that such cell-seeded constructs would demonstrate a cartilage-like morphology with expression of chondrocyte-specific molecules, while also conserving sufficient biomechanical characteristics, after 12 weeks *in vivo*.

Materials and methods

Isolation, culture and induction of myoblasts. Canine myoblasts were isolated and cultured as previously described (10). Primary cells were seeded on a culture dish at a density of $5x10^5$ cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco-BRL), 300 μg/ml of L-glutamine, 50 μg/ml vitamin C, 100 U/ml penicillin G, 100 μg/ml streptomycin and amphotericin B 0.25 μg/ml (all from Sigma, St. Louis, MO, USA). After medium change, cultured myoblasts were subjected to chondrogenic induction with culture medium containing 50 ng/ml CDMP-2 and 20 ng/ml TGF-β1 (Sigma). The myoblasts were cultured at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed every third day, and this washed out all non-adherent cells. Cells were subcultured at a density of $1.0x10^4$ cells/cm^2 and treated with 0.25% trypsin

Correspondence to: Dr Guoxing Zhu, Department of Orthopedics, Wuxi No. 2 People's Hospital, 68 Zhongshan Road, Wuxi, Jiangsu 214002, P.R. China
E-mail: guoxing.zhu@yahoo.cn

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plus 0.02% EDTA (Gibco-BRL) when they reached 80% confluence. The study was approved by the ethics committee of Tongji University, Shanghai, China.

Immunocytochemistry assay of collagen II. To determine the *in vitro* chondrogenic induction effect, induced myoblasts were examined for type II collagen expression using immunocytochemical staining. Briefly, cells were incubated at 37˚C for 1 h with mouse anti-collagen-II monoclonal antibody (IgG1; BD Biosciences Clontech, Franklin Lakes, NJ, USA) diluted in phosphate-buffered saline (PBS, 1:200), followed by incubation with 1:100 diluted horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Dako, Carpinteria, CA, USA) for 30 min and color development with diaminobenzidine tetrahydrochloride (DAB). Normal menisci served as positive controls.

Preparation of PLGA scaffold and cell seeding. A non-woven copolymer scaffold of L-lactide and glycolide (90/10, PLGA) in the form of fibers was generously provided by Shanghai Ju Rui Biomaterials Co., Inc., (China). The scaffolds were cylindrical, with a diameter of 10 mm and a thickness of 2 mm. The pore sizes of the non-woven fibers were on average 75 μm, the pore volume accounted for 97% of the total volume, and the filament diameter was 13 μm. The PLGA constructs were treated using the low-pressure plasma technique at the end of the production process. A partially ionized gas reacted with the surface of the scaffolds and formed reactive particles. Prior to cell seeding, the scaffolds were immersed in DMEM/F12 medium containing 10% (v/v) FBS for 12 h to enhance cell adhesion onto the scaffold.

Chondrogenically induced myoblasts at passage 3 ($1.5x10^7$ in 0.3 ml) were harvested and placed onto PLGA scaffolds, respectively, to form cell-scaffold constructs and the constructs were cultured at 37˚C in a humidified atmosphere of 5% CO_2 for 5 h, which allowed the complete adhesion of myoblasts to the scaffold. The cell-PLGA constructs in inductive media were subsequently cultured *in vitro* for 14 days. Medium was changed three times a week. As an experimental control, scaffolds with non-induced myoblasts were cultured for the same lengths of time.

Surgical procedure. Six-week-old athymic nude mice were used in this study, and were obtained from the Agricultural Institute of Shanghai Jiaotong University, China. Animal care and experimental procedures were in accordance with the guidelines of the Administrative Panel on Laboratory Animal Care of China. Under general intraperitoneal anesthesia and after disinfection of the back of each of the 24 mice, two subcutaneous pockets were bluntly created through a 1.5-cm incision in the back. A construct (induced cell-PLGA construct or non-induced cell-PLGA construct control) was inserted in each subcutaneous pocket. The wound was closed using a single interrupted suture. No animal died during the experimental period. The animals were sacrificed and the implants were harvested at 8 and 12 weeks, respectively.

Histological and immunohistochemical analyses. Eight and 12 weeks after implantation, the implants were retrieved and analyzed histologically and immunohistochemically, respectively. For histological analyses, specimens were fixed in 10% (v/v) buffered formalin, dehydrated with a series of graded alcohol and embedded in paraffin. Tissue sections (4-μm thick) were stained with hematoxylin and eosin for morphological analysis.

Expression of collagen type II was detected using monoclonal antibodies (Dako). Briefly, after deparaffinization, sections were predigested with trypsin at 37˚C for 30 min to facilitate antibody access, endogenous peroxidase was removed by the treatment of 0.3% H_2O_2 in methanol at room temperature for 30 min, and non-specific antibody binding was blocked by incubation of sections in 10% normal goat serum at 37˚C for 30 min. Mouse anti-canine collagen type II diluted 1:100 in 0.01 M PBS (pH 7.4) was applied as a primary antibody at 4˚C overnight. Sections were then incubated with the secondary antibody, rabbit anti-mouse immunoglobulin (Dako) for 60 min, followed by application of mouse PAP kit (Dako). Collagen type II was visualized by the reactions with 0.05% diaminobenzidine containing 0.01% H_2O_2.

Analysis of mRNA for extracellular matrices and collagen with reverse transcriptase-polymerase chain reaction. RNA samples were obtained after 12 weeks and transcribed into cDNA by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for gene expression of type I collagen, type II collagen and aggrecan. After explantation, the constructs were digested with Ultraturax™ and total mRNA was prepared using TRIzol reagent according to the manufacturer's instructions (Gibco-BRL). Total RNA (1 μg) was treated with 1 unit of deoxyribonuclease I (DNase I; Gibco-BRL) in order to digest genomic DNA contamination. Random-primed cDNA synthesis was performed using 1 μg of DNase I-treated total RNA and 50 units of StrataScript reverse transcriptase according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). TaqMan™ PCR assays were performed in 96-well optical plates on an ABI Prism 7700 Sequence Detection system (Applied Biosystems, Forster City, CA, USA) using ABsolute QPCR ROX Mix (Abgene, Hamburg, Germany) according to the manufacturer's instructions. The thermal cycling conditions were 95˚C for 15 min followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. The PCR products were analyzed by electrophoresis in 2% agarose gels and stained with ethidium bromide. The mRNAs analyzed were collagen I (681 bp), collagen II (447 bp), aggrecan (321 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (211 bp). Primer sequences for GAPDH, aggrecan, collagen I and collagen II were as follows: GAPDH, sense 5'-CCTCTATGCCAACACAGTGC-3' and antisense 5'-GTACTCCTGCTTGCTGATCC-3'; aggrecan, sense 5'-TAG AGAAGAAGAGGGGTTAGG-3' and antisense 5'-AGCACT AGGAGCCAGGGTTAT-3'; collagen I, sense 5'-ATGCCC AAGACTACCAGTGG-3' and antisense 5'-TCCTGG AAGCTCTTCTCAGT-3'; collagen II, sense 5'-TTTCCC AGGTCAAGATGGTC-3' and antisense 5'-CTTCAGCAC CTGTCTCACCA-3'.

Biomechanical analysis of engineered cartilage. Eight and 12 weeks after implantation, the biomechanical properties of the engineered cartilage were tested by measuring the compressive modulus. Briefly, the specimens were harvested and trimmed

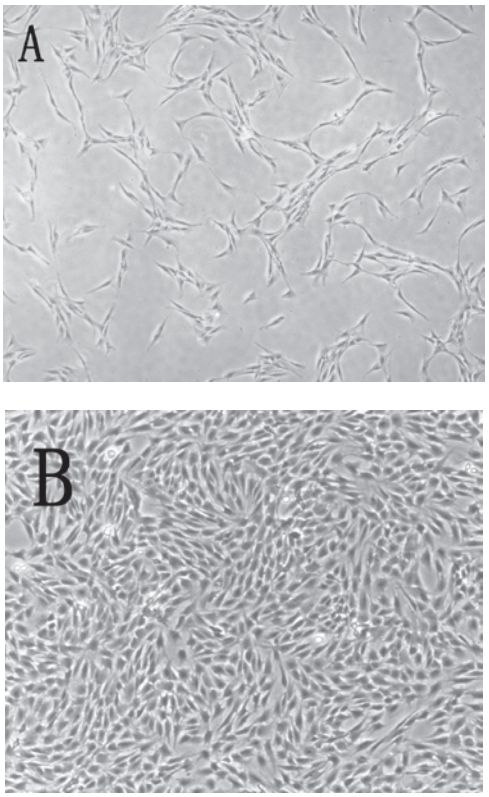

Figure 1. Morphological change between primary myoblasts and chondrogenically induced myoblasts. (A) Morphology of canine myoblasts cultured in a monolayer manner on day 3 in primary culture. (B) Myoblasts undergo chondrogenic differentiation after 14 days. Magnification, 100 μm.

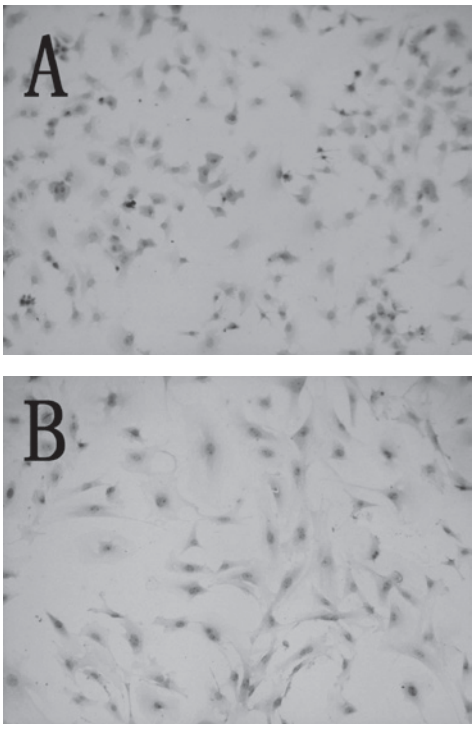

Figure 2. Immunocytochemical staining demonstrates a stronger expression of collagen II in (A) induced cells than in (B) non-induced cells. Magnification, 200 μm.

to fit in a test chamber (5 mm diameter) of a biomechanical analyzer (Instron, Canton, MA, USA). A constant compressive strain rate of 1 mm/min was used until the maximal force of 450 N was reached, and thus a force-displacement curve was obtained. The compressive moduli of tested tissues were automatically calculated by the machine and further verified by manual calculation with the formula: $\Delta P/A$ x $L/\Delta L$ (ΔP, the compressive force margin of the 2 points on the linear segment of the load-displacement curve before the first break point; ΔL, the displacement margin of the corresponding two above-mentioned points; A, the area of tested tissue; L, the thickness of tested tissue). In addition, the normal canine menisci were tested as a control. ANOVA analysis was applied and $P<0.05$ was considered to indicate a statistically significant result.

Glycosaminoglycan (GAG) quantification of engineered cartilage. GAG quantitative analysis of 8- and 12-week specimens was performed. Briefly, the specimens were minced and triturated to prepare the protein solution. A series of reagents was added step by step to ensure the specific binding of Alcian blue and polysulfated molecules of GAGs in engineered cartilage. All GAGs were precipitated specifically in guanidine-HCl by using a low pH in combination with detergent and a high salt concentration. The precipitate was dissolved in a mixture of guanidine-HCl and propanol. For quantification, the absorbance was recorded in a microplate reader with a 600-nm filter, and a linear standard curve between 0.5 and 20 μg was generated by adding known amounts of proteoglycans. The results were analyzed using the ANOVA test. A P-value <0.05 was considered statistically significant. Since no neocartilage was generated we did not analyse the quantitive GAG in the control group. The positive control was also tested as a normal meniscus.

Statistical analysis. The presence of type II collagen in the repaired meniscus tissue from the different groups was noted and statistically compared using the Fisher's exact test. Compressive modulus and GAG content in the different groups were analyzed with ANOVA. For all evaluations, the level of statistical significance was set at a probability value of <0.05.

Results

Morphological and immunohistochemical analysis. As shown in Fig. 1, myoblasts induced with CDMP-2 and TGF-β1 underwent a morphological change after chondrogenic induction, approaching the shape of native chondrocytes. In addition, the induced cells showed significantly enhanced collagen II expression compared to control cells (Fig. 2).

Gross morphology of engineered cartilage. Specimens harvested after 8 weeks of *in vivo* culture showed that all induced cell-PLGA constructs maintained the approximate original scaffold shape and size, and had a compact consistency (Fig. 3). The non-induced cell-PLGA constructs showed a failure to maintain the shape and size of the original scaffold. Twelve-week specimens showed a more cartilaginous appearance. However, no construct was identified in the control group, indicating that the scaffold was degraded completely at 12 weeks.

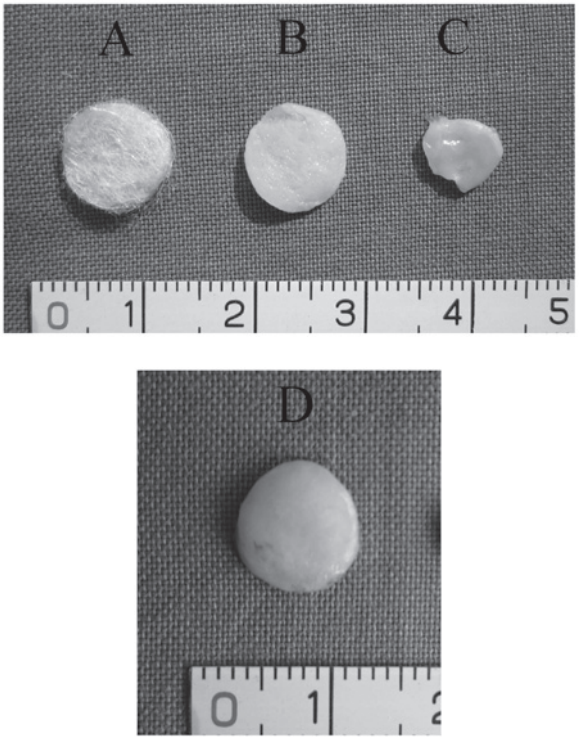

Figure 3. Gross morphology of engineered cartilage. (A) A PLGA scaffold, (B) an induced cell-PLGA construct at 8 weeks post-implantation, (C) a non-induced cell-PLGA construct at 8 weeks post-implantation and (D) an induced cell-PLGA construct at 12 weeks post-implantation. PLGA, poly-lactide-co-glycolide.

Histological and immunohistochemical assessment of engineered cartilage. A histological section of engineered cartilage at 8 weeks had the appearance of fibrocartilage with fibrochondrocytic-like cells (Fig. 4A). In the control group, only a band of fibrous tissue was observed (Fig. 4B). The engineered cartilage at 12 weeks had histological structures more similar to those of normal cartilage than those at 8 weeks (Fig. 4C).

Immunohistological staining indicated that engineered cartilage was positively stained for type II collagen at 8 weeks post-implantation (Fig. 5A). With the maturation of engineered cartilage, the expression and distribution of collagen II was found to be similar to that of the neighboring native meniscus at 12 weeks post-implantation (Fig. 5C). However, no expression of collagen II was observed in all specimens of the control group at 8 weeks (Fig. 5B).

Expression of cartilage-specific genes by RT-PCR. To investigate the chondrogenic differentiation of the engineered cartilage, mRNA expression of type I collagen, type II collagen and aggrecan was assessed using RT-PCR. At 12 weeks, expression of mRNAs for type I collagen, type II collagen and aggrecan was detected. By contrast, none of the assessed genes were expressed in the control tissue (Fig. 6). Gene expression of the normal meniscus was assessed as a positive control.

Biomechanical properties of engineered cartilage. As summarized in Table I, the compressive moduli increased with length of transplantation time. At 8 weeks, the moduli reached

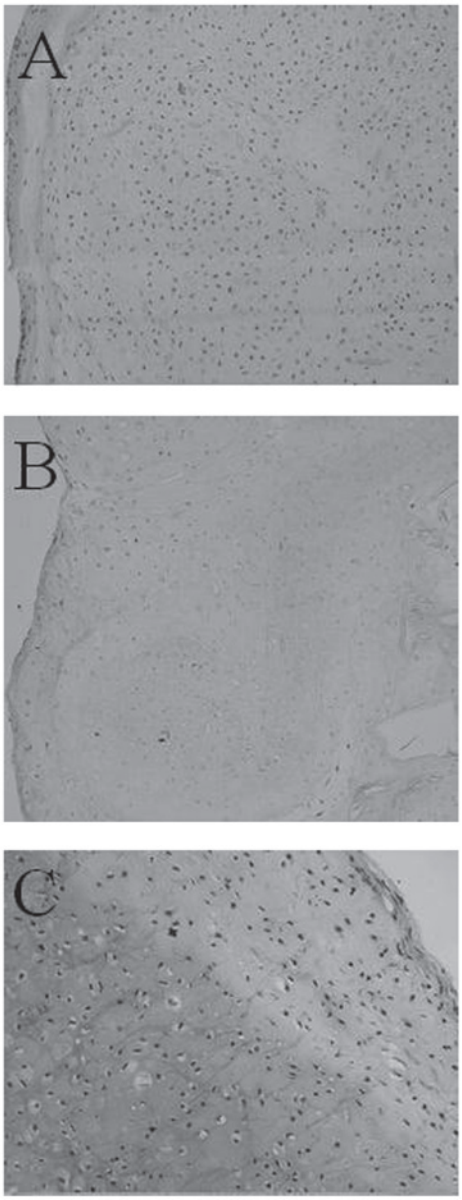

Figure 4. Hematoxylin and eosin histology of engineered cartilage. (A) An induced cell-PLGA construct at 8 weeks post-implantation, (B) a non-induced cell-PLGA construct at 8 weeks post-implantation and (C) an induced cell-PLGA construct at 12 weeks post-implantation. PLGA, poly-lactide-co-glycolide.

50.41 and 23.16% of the normal meniscus level, respectively, in the experimental and control groups, and the experimental group increased further to 85.72% at 12 weeks. Statistical analysis demonstrated that the compressive modulus was higher in the induced group (Exp) than in the non-induced group (Ctrl) ($P<0.05$), indicating that *in vitro* chondrogenic induction is helpful for improving biomechanical properties of myoblast-engineered cartilage.

GAG content quantification. The GAG deposition of the engineered cartilage was further quantified by biochemical analysis and also compared with that of the normal meniscus. As shown in Table II, it was found that the amount of GAG in the experimental group at 8 weeks reached 47.39% of that in the normal meniscus, as compared with 23.26% for

Table I. The compressive moduli of repaired cartilage.

Groups	8 weeks (n=12)		12 weeks (n=12)	
	Moduli (MPa)	Percentage of normal (%)	Moduli (MPa)	Percentage of normal (%)
Normal	27.12±0.69		28.92±1.35	
Exp	13.67±1.830	50.41±2.93	24.79±2.78	85.72±4.23
Ctrl	6.28±1.52	23.16±2.49		

The compressive moduli increased with the transplanted time and the value is always higher in induced cell-PLGA constructs group (Exp) than in non-induced cell-PLGA constructs group (Ctrl) with a statistically significant difference (P<0.05).

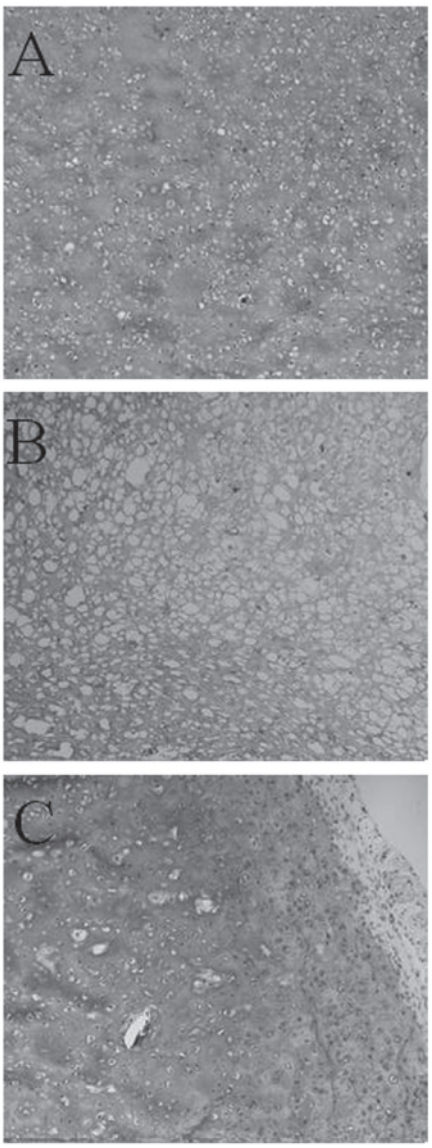

Figure 5. Type II collagen immunohistochemistry of engineered cartilage. (A) An induced cell-PLGA construct at 8 weeks post-implantation, (B) a non-induced cell-PLGA construct at 8 weeks post-implantation and (C) an induced cell-PLGA construct at 12 weeks post-implantation. PLGA, poly-lactide-co-glycolide.

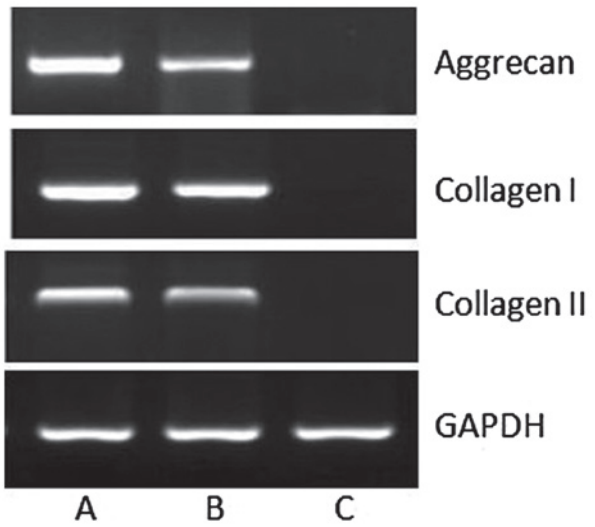

Figure 6. Analysis of expression of mRNA for aggrecan, collagen I, II and GAPDH with RT-PCR. (A) Positive control using a normal meniscus. (B) An induced cell-PLGA construct 12 weeks post-implantation and (C) a non-induced cell-PLGA construct 12 weeks post-implantation. PLGA, poly-lactide-co-glycolide.

the control group (P<0.05). However, with the maturation of the engineered cartilage to 12 weeks, GAG content was increased, reaching 91.35% of that in the normal meniscus (P<0.05). A significant difference was also observed between the amount of GAG in the engineered cartilage at 8 and 12 weeks (P<0.05).

Discussion

Meniscal injury is one of the most common injuries to the knee. The menisci are important for normal knee function. A meniscus injury increases the risk of subsequent development of degenerative changes in the knee (11). Repairing meniscus injuries remains a challenge in sports medicine, since meniscus usually does not regenerate by itself following injury. The emergence of a tissue engineering technique may provide a satisfactory solution to the problem.

Tissue engineering is based on a unique combination of exogenous cells, specific stimuli and matrix scaffold in an *in vitro* or *in vivo* environment. In the present study, myoblasts were used. Myoblasts, which are adult stem cells and include skeletal muscle cell precursors, have been shown to possess multipotential mesenchymal stem cell activity and are capable of forming chondrocytes, osteocytes and adipocytes as well as myocytes (12-14). Previous reports suggest that

Table II. GAG contents (wet weight) of repaired cartilage.

	8 weeks (n=12)		12 weeks (n=12)	
Groups	GAG content (mg/g)	Percentage of normal (%)	GAG content (mg/g)	Percentage of normal (%)
Normal	12.07±0.37		12.95±1.23	
Exp	5.72±0.87	47.39±2.37	11.83±3.05	91.35±5.27
Ctrl	2.81±1.03	23.26±4.76		

GAG content is higher in Exp than in Ctrl ($P<0.05$) and no significant difference was found between the normal and experimental groups ($P>0.05$). GAG, glycosaminoglycan.

a degree of plasticity remains prior to terminal myoblastic differentiation. Therefore, myoblasts may prove useful for the development of new therapeutic approaches aimed at the regeneration of damaged or diseased tissues. Myoblasts have been investigated as a candidate cell source for tissue engineering (15-17). In comparison with other stem cell sources, myoblasts represent a more promising source for cartilage engineering, as they are relatively abundant and easily accessible, with minimal donor site morbidity (18,19). Studies have shown that myoblasts have a higher cell yield and more rapid proliferation ability during *in vitro* expansion (8,20). In this study, we observed a chondrogenic response with a dose of 50 ng/ml CDMP-2 and 20 ng/ml TGF-β1 provided continuously with each medium change. Chondrogenic differentiation, analyzed using immunohistochemistry and gene expression profiles, was observed. This suggests that key events responsible for the commitment of myoblasts to the chondrogenic lineage occur during the early initial period of cell growth and proliferation.

PGA is a commonly used synthetic polymer in cartilage tissue engineering. In order to maintain its dimensional stability and enhance its mechanical properties, fibrous PGA meshes are coated with solutions of PLA. Evaporation of the solvent for PLA leads to the formation of PLGA composites with specific shapes. The feasibility of using a PLGA composite as a scaffold to engineer cartilage tissue has been documented in a number of studies (21,22). It was also shown that adhesion and proliferation of chondrocytes on PGA fibers was significantly suppressed when an increased amount of PLA was added (21,22). Therefore, the concentration of PLA solution to be added needs to be lowered but still sufficient to function as glue to maintain the structural stability of the PGA 3D scaffold. In the present study, 1.5% PLA in dichloromethane was used. Any further lowering would cause an unstable configuration of the scaffold. Scanning electron microscopy revealed that PLA at this concentration is capable of wrapping PGA fibers together and that the shape of scaffold was maintained when they were kept in culture medium for as long as 5 weeks (22).

The main advantage of using a PLGA scaffold for meniscal tissue engineering is its suitable degradation rate, which matches the kinetics of new meniscal formation *in vivo* (5). The degradation of non-woven PGA scaffold is reported to be complete over a period of 2 months *in vivo* (23). In the present study, no undegraded PLGA fibers were observed histologically in the experimental or control groups at 12 weeks post-implantation. Due to its rapid degradation, PLGA scaffold was found to accelerate chondrogenesis of constructs prepared from dedifferentiated chondrocytes and PLGA, as the accumulation of the deposited cartilage-specific extracellular matrix (ECM) and expression of marker genes both *in vitro* and *in vivo* were significantly enhanced compared with those of constructs prepared from PGA. It was also proposed that early degradation of PLGA fibers may have a positive effect on chondrogenesis by leaving new spaces for cells to further fill in and produce new intercellular matrix, which in turn may facilitate the formation of more cell-matrix and cell-cell contact.

In this study, the specimens showed a cartilaginous appearance. However, no construct was identified in all control groups after 8 weeks of *in vivo* culture. As shown by the histological analysis, the section of engineered cartilage had the appearance of fibrocartilage with fibrochondrocytic-like cells at 8 and 12 weeks. In the control group, only a band of fibrous tissue was observed. Immunohistological staining indicated that engineered cartilage was positively stained for type II collagen compared with the control group at 8 and 12 weeks post-implantation. At 12 weeks, expression of mRNAs for type I collagen, type II collagen and aggrecan was detected by RT-PCR. By contrast, no assessed genes were expressed in the control tissue. In addition, the biomechanical properties (compressive modulus) and biochemical composition (GAG quantification) of engineered cartilage at 12 weeks post-implantation were similar to those of corresponding normal cartilage, which indicated that the engineered cartilage was formed both structurally and functionally.

In conclusion, we showed chondrogenic differentiation of myoblasts seeded into PLGA scaffolds following implantation in a subcutaneous pocket of nude mice. Further studies are required to investigate implanted myoblast-seeded PLGA scaffolds in a joint, including biomechanical stress for the evaluation of a possible positive stimulus by mechanical loading after implantation.

Acknowledgements

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