Yonsei Medical Journal
Vol. 45, Suppl., pp. 41 - 47, 2004

Chondrogenic Differentiation of Mesenchymal Stem Cells and Its Clinical Applications

Jin Woo Lee[1,2], Yun Hee Kim[1,2], Su-Hyang Kim[1,2], Seung Hwan Han[1], and Soo Bong Hahn[1]

[1]Department of Orthopaedic Surgery, Yonsei University College of Medicine, Seoul, Korea;
[2]Brain Korea 21 Project for Medical Science, Yonsei University, Seoul, Korea.

Tissue engineering has the potential to provide cartilaginous constructs capable of restoring the normal function of native articular cartilage following joint injury or degradation. One approach to functional tissue engineering of cartilage involves the *in vitro* cultivation of tissue constructs by using: (i) chondrogenic cells that can be selected, expanded, and transfected to overexpress the genes of interest, (ii) scaffolds that provide a defined three-dimensional structure for tissue development and biodegrade at a controlled rate. Understanding the functional potential of the cells and the signaling mechanisms underlying their differentiation should lead to innovative protocols for clinical orthopaedic interventions. A large number of growth factors and hormones have been implicated in the regulation of chondrocyte biology, relatively little is known about the intracellular signaling pathways involved. We have tried to define the roles of specific TGF-β dependent signaling pathways involved in the regulation of chondrogenesis from human mesenchymal stem cells. Chondrogenesis induced by TGF-β_3 in alginate bead system was confirmed by examining cartilage specific type II collagen expression and aggrecan, whereas type I collagen expression was not affected by TGF-β_3. Type II collagen mRNA expression was expressed strongly during chondrogenesis and MEK inhibition (U0126) resulted in complete down-regulation of type II collagen. In contrast, aggrecan expression was detected in same level by treatment of U0126. These results strongly suggest that the ERK signaling cascade is involved in TGF-β_3 induced-chondrogenesis signaling pathways and a role of its pathway is necessary over a longer period to promote type II collagen expression. However, their end product properties *in vivo* have not been well known. In this study, an articular cartilage from chondrogenic MSCs with PLGA scaffolds (75:25 and 65:35) were made and analyzed its biochemical, histological and mechanical properties *in vitro* and *in vivo*. And also, we evaluated the cartilage formation *in vivo* through the injection of cell-thermosensitive gel complex, a newly developed injectable material. At 12 weeks after PLGA scaffolds containing chondrogenic MSCs transplantation, the separated rabbit distal femur showed a good gross articular cartilage appearance in the transplanted site. In indentation test, compare to the native articular cartilage, the engineered cartilage from two types of (75:25 and 65:35) achieved up to 30-60% in mechanical stiffness. And also, a new model for cartilage formation in bladder, at 14 weeks after injection, we could find out mass formation in the submucosal area grossly. H&E staining, alcian blue staining and other special staining confirmed the chondrogenic differentiation in the mass. These cell therapy technologies can provide the possibility of clinical applications for vesicoureteral reflux and reflux esophagitis, and urinary incontinence as well as articular cartilage regeneration.

Key Words: Mesenchymal stem cell, chondrocyte, TGF-β_3 signaling, PLGA scaffold, thermosensitive gel

Bone marrow mesenchymal stem cells (MSCs) are multipotent cells, which can differentiate into osteoblasts, chondrocytes, adipocytes, and so on. These cells may be isolated from the marrow using standardized techniques and expanded in culture, while maintaining their capacity to differentiate along these pathways when showed to appropriate culture conditions.[1,2] This property of mesenchyaml stem cells helps therapeutic opportunities for the treatment of damages in mesenchymal tissues.[1,3,4] To improve the repair of bone and cartilage, several modalities such as bone

Received January 30, 2004

This research was supported by a grant (code SC13142) from Stem Cell Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea.

Reprint address: requests to Jin Woo Lee, M.D., Ph.D., Department of Orthopaedic Surgery, Yonsei University College of Medicine, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-752, Korea. Tel: 82-2-361-5651, Fax: 82-2-363-1139, E-mail: ljwos@yumc.yonsei.ac.kr

marrow alone, bone marrow-scaffold composites, and culture expanded MSC implantation have been tried.[5-7]

Two representative culture systems have been developed that promote the chondrogenic differentiation of human marrow mesenchymal progenitor cells. These systems are adaptations of the "pellet" culture system that was originally described as a method for preventing the phenotypic modulation of chondrocytes and "alginate bead" culture system that maintained encapsulated cells as their differentiated phenotype over time.[3,8-11] More recently, the "pellet" culture has been used in studies of the terminal differentiation of growth-plate chondrocytes. This culture system allows cell-cell interactions analogous to those that occur in precartilage condensation during embryonic development. Also, freshly isolated articular chondrocytes cultured in "alginate beads" for 2 weeks synthesize a matrix similar to that of native articular cartilage and maintain their phenotype for as long as eight months and they have been shown that culturing of dedifferentiated chondrocytes in alginate induces re-expression of the differentiated chondrocyte phenotype.[3,6,12,13] However, this cell configuration is not sufficient for the induction of chondrogenesis: the chondrogenic differentiation of the marrow-derived progenitor cells required the use of a defined medium to which were added certain bioactive factors, including insulin, transferrin, selenium, pyruvate and TGF-β.[3,6,14] Members of the transforming growth factor-β (TGF-β) family of growth factors have been shown to play a major role in bone and cartilage development. TGF-β evokes a chondrogenic response in embryonic mesenchymal cells, and periosteum-derived cells. Several studies have shown that TGF-β isoforms differ in their effects on chondroprogenitor and other cells.[14] Transforming growth factor-β (TGF-β) can induce chondrogenic differentiation of mouse or human bone marrow stromal cells, as judged by expression of aggrecan and type II, IX and X collagen. These systems provided means for studying the process of chondrogenesis, including those factors that regulate the progression of cells through the entire chondrogenic lineage.

Regulation of Chondrogenesis

Recent studies have demonstrated that TGF-β, BMP-2, and growth differentiation factor-5 (GDF-5) rapidly induce type II collagen expression, suggesting critical roles of signaling by the TGF-β superfamily for chondrocyte-specific gene expression.[15-17] Although the Smad pathway is widely represented in most of the cell types and tissue studied,[18] additional pathways may be activated following treatment with TGF-β in specific contexts. Activation of Ras, extracellular signal-regulated kinase 1/2 (ERK1/2), and c-Jun N-terminal kinase (JNK) by TGF-β signaling has been reported in primary intestinal epithelial cells and chondrogenic cell line derived from mouse teratocarcinoma.[19,20] We have tried to define the contribution of specific TGF-β dependent signaling pathways involved in the regulation of chondrogenesis from human mesenchymal stem cells. To inhibit the ERK1/2 pathway, we used U0126, a specific inhibitor of mitogen activated protein kinase 1/2 (MEK1/2), which is an upstream molecule that activates ERK1/2. For inhibition studies, U0126 was used at the concentration indicated and was added with TGF-β_3 treatment at the same time. TGF-β_3 induced phosphorylaion of ERK1/2 at 1 hour and reached the peak at 10 hour. Chondrogenesis induced by TGF-β_3 in alginate bead system was confirmed by examining cartilage specific type II collagen expression and aggrecan, whereas type I collagen expression was not affected by TGF-β_3. Type II collagen mRNA expression was expressed strongly during chondrogenesis and MEK inhibition resulted in complete down-regulation of type II collagen. In contrast, aggrecan expression was detected in same level by treatment of U0126. It seemed that type II collagen expression might be critically regulated by downstream signaling molecules of ERK1/2 (Fig. 1A).

The ERK1/2 activation by TGF-β_3 during chondrogenesis was showed weakly at 1 hour and reached the peak at from 10 hour to 1 day (Fig. 1B). The ERK1/2 activation was maintained until 14 days. This result showed that TGF-β_3 induced-chondrogenesis was regulated through ERK1/2 signaling pathway. Because treatment with MEK inhibitor showed complete ERK

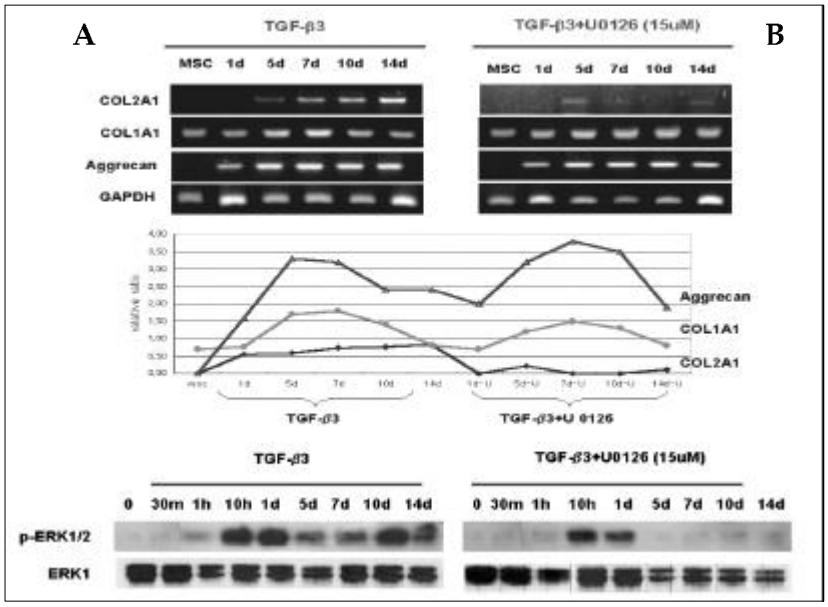

Fig. 1. RT-PCR analysis during chondrogenesis. After treatment of U0126, type II collagen expression by aggrecan was completely inhibited for 14 days (A). Western blot analysis. ERK1/2 activation was maintained at all time points stimulated by TGF-β_3 during chondrogenesis from human mesenchymal stem cells (B).

inactivation at all timepoints except at 10 hour and 1 day. Comparing with gene expression, it suggested that ERK1/2 mediated signaling pathway might be one of the key signaling factors and its inactivation resulted in blocking of chondrogenesis from human mesenchymal stem cells. Chondrogenesis of mesenchymal stem cell is regulated by complicated protein kinase signaling cascades. We demonstrated that the inhibitory effects of MEK inhibitor on chondrogenesis only showed down-regulation of type II collagen. Recently, it has been reported that aggrecan gene expression was regulated by cross-talk between Smad, ERK1/2, and p38 mitogen-activated protein kinase (MAPK) pathway but not protein kinase A.[21] Therefore, we thought that aggrecan expression was not down-regulated by treatment of MEK inhibitor unlike type II collagen. These results strongly suggest that the ERK signaling cascade is involved in TGF-β_3 induced-chondrogenesis signaling pathways and a role of its pathway is necessary over a longer period to promote type II collagen expression.

Regeneration of Articular Cartilage Using Mesenchymal Stem Cells

Recently, a new tissue engineering using the concept of cell therapy has been developed to promote cartilage regeneration including the autologous chondrocyte transplantation.[22,23] However, some problems have been brought up, such as an uneven distribution of the grafted chondrocytes through the cartilage defects and a high risk of the leakage of grafted chondrocytes that form the defects. In order to overcome an uneven distribution and the leakage of chondrocytes, researchers have developed the polymeric scaffolds and found new cell source, mesenchymal stem cells.[1,24,25] The chondrogenic potentials of MSCs have attracted considerable interests and many studies have been done. However, their end product properties are not well known. In this study, an articular cartilage from chondrogenic MSCs with polymeric scaffold were made and analyzed its biochemical, histological and mechanical properties *in vitro* and *in vivo*. The cells obtained from New Zealand white rabbit bone marrow aspirates were first isolated by monolayer culture. After 4 weeks of monolayer culture, the cells were seeded into the polylactic acid-co glycolic acid (PLGA) scaffolds (two types of PLGA; 75:25, 65:35). The cells were cultured in PLGA scaffold for 21 days in a defined medium consisted of TGF-β_3 (10ng/ml) and ascorbic acid(50μg/ml) on a rotator in a 5% CO_2 incubator at 37℃. Before transplanting to the rabbit distal femur, the chondrogenesis of MSCs-seeded polymer scaffolds was confirmed by histology and RT-PCR. The type II collagen and

aggrecan gene expression were detected by RT-PCR. Furthermore, the amount of collagen and glycosaminoglycan in the medium of the three-dimensional culture during chondrogenesis was measured for the proper timing of transplantation. To assess histological properties, routine H & E staining and safranine O staining was used. After biochemical and histological analysis, chondrogenic MSCs-seeded polymers were transplanted to the rabbit autologously. In the patellar groove of femur, the defect of the same size of scaffold was gently prepared aseptically, and chondrogenic MSCs-seeded polymer was grafted with fibrin glue (Tissel, 3M). At 12 weeks after operation, the distal femur of the rabbit was separated after euthanasia. Initially, the gross morphology of the regenerated articular cartilage was assessed, and their mechanical properties were measured using the indentation test. Testing was carried out in displacement control (50 μm) with the speed of 10 μm/sec. The diameter of the indentor was selected to be 1 mm. The center and interface site of regenerated cartilage were checked. The Young's modulus was determined using the equation derived from the solution of Hayes et al. (1972) for single phasic elastic material. The thickness of cartilage was measured from the histological sections. The determination of the last product was performed using H&E and safranine O staining and histological scoring was carried out (Fig. 2). After 3 weeks of three-dimensional (3D) culture, the RT-PCR demonstrated that type II collagen and aggrecan was well expressed with the control analysis of normal articular chondrocyte of rabbit.

The total collagen secretion started after 14 days and showed a gradual increase to the end of the 3D culture days. Furthermore, GAG excretion started after 17 days of culture and increased with time, and thus it was confirmed that the 3 weeks of 3D culture was a proper time for transplantation. In histology, MSCs were well attached to the polymeric fibres and some of extracellular matrix was well noted. In addition, GAG and type II collagen was well expressed in the safranine O. The separated rabbit distal femur showed a good gross articular cartilage appearance in the transplanted site. The morphological scores showed no significant difference statistically when compared with the normal articular cartilage. In indentation test, compare to the native articular cartilage, the engineered cartilage from two types of PLGA scaffolds (75:25 and 65:35) achieved up to 30-60% in mechanical stiffness. Also, compared to the fibrous healing sample, it was more flexible. In histology, all of them well expressed hyaline-like cartilage. Their histological scores statistically had no differences from the normal control samples of sham operation, but slightly decreased. When compared to the defect fibrosis control sample, scores of the engineered cartilages were statistically high. In conclusion, it showed that chondrogenic MSCs seeded with biodegradable porous

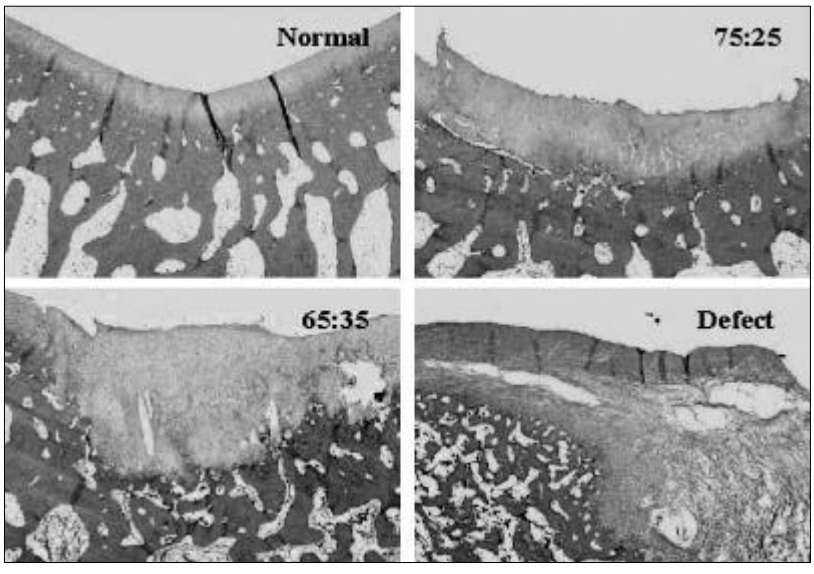

Fig. 2. Histological analysis with H&E stain at 12 weeks after operation. In histology, MSCs were well attached to the polymeric fibres and some of extra cellular matrix was well noted.

polymeric scaffolds are the new source for the treatment of articular cartilage defects with no difference in mechanical and histological properties.

The Other Clinical Applications of Chondrogenic Mesenchymal Stem Cells with a Novel Liquid Polymer

Tissue engineering using mesenchymal stem cells (MSCs) continues to be challenging problem. Using these cells, therapeutics as a minimally invasive technique for ureteral reflux or reflux esophagitis have been tried.[26-29] Poly-N-isopropylacrylamide (PNIPAAm) is well known to have thermo-sensitivity and has the lower critical solution temperature (LCST, 32℃) at which a macromolecular transition from a hydrophilic to a hydrophobic structure occurs.[30] Chitosan is one of the useful natural polymeric biomaterials due to its biocompatibility and biodegradable property. It has good characters for cell attachment, proliferation and viability.[31-33] We assessed the ability to differentiate from MSCs to chondrocytes and mass formation using a newly developed injectable material, the thermosensitive (water soluble chitosan-g-NIPAAm) gels, and evaluate the cartilage formation *in vivo* through the injection of cell-thermosensitive gel complex. We observed cell cultures in WSC-g-PNIPAAm and alginate beads. The cell viability of both groups showed no significant differences at days 5 and 10. The level of aggrecan expression in WSC-g-NIPAAm copolymer gel showed more gradual increase than that in alginate bead culture. And the expression of type II, and X collagen in thermosensitive gel culture were increased but that of type I collagen was decreased or maintained at a uniform level similar to those in alginate gel culture. WSC-g-PNIPAAm and MSCs complex were injected in the submucosal layer of bladder of six rabbits. The rabbit bladder was exposed by open surgery and then the submucosal injection was done in the dome area. At 14 weeks after injection, we could find out mass formation in the submucosal area grossly. The mass was rubber like feeling by palpation. H&E staining, alcian blue staining and other special staining confirmed the chondrogenic differentiation in the mass (Fig. 3). There were small round cell with lacuna formation. These lacunae were relatively smaller than normal articular cartilage tissue.

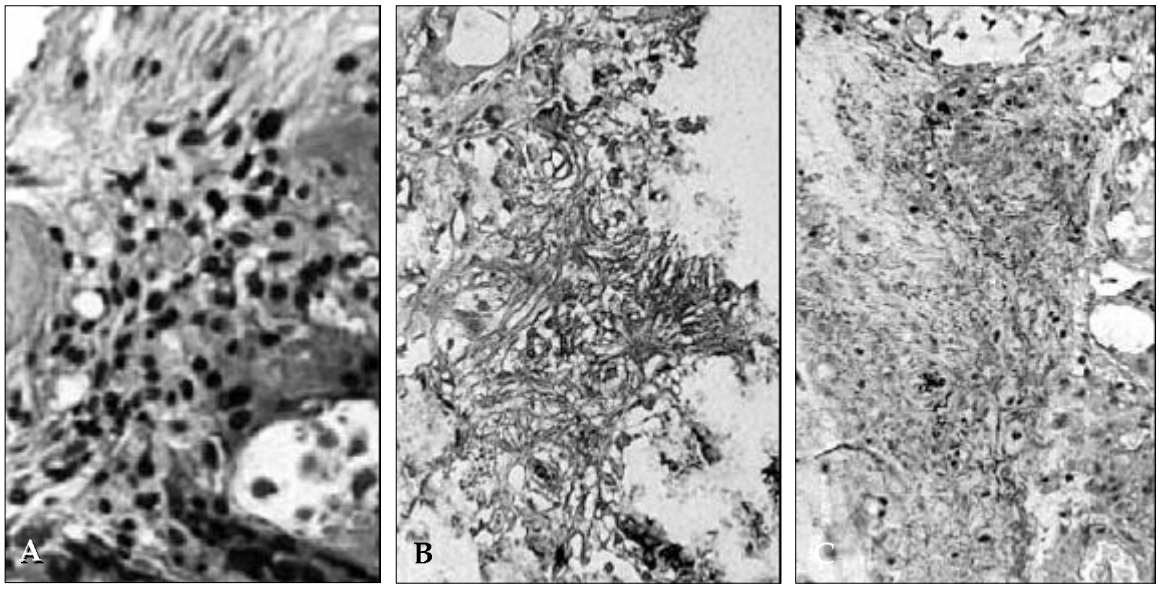

Fig. 3. Hematoxylin-eosin (A) and immunohistochemical staining for collagen type I (B) and II (C) findings of the mass in the injected area (A: Hematoxylin-eosin stain showed a cellular area having cytologic features of abortive lacunae formations. B: Immunohistochemical staining for collagen type I showed pericellular collagen type I deposition. C: Immunohistochemical staining for collagen type II showed pericellular collagen type I deposition. It is characteristics of hyaline cartilage).

A novel WSC-g-PNIPAAm copolymer with chondrogenic MSCs showed the possibility of clinical applications especially such as cell therapy technologies for vesicoureteral reflux and reflux esophagitis, and urinary incontinence.

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