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Original Article

Gene and protein expressions of bone marrow mesenchymal stem cells in a bone tunnel for tendon-bone healing

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ABSTRACT

Purpose: Bone marrow mesenchymal stem cells (BMSCs) injected around tendon grafts can enhance tendon-bone healing and promote fibrocartilage formation. To understand gene and protein expressions of cells during tendon-bone healing, auto-BMSCs were implanted into a bone tunnel in anterior cruciate ligament reconstruction in a rabbit model.

Methods: BMSCs were harvested from New Zealand white rabbits. By an anterior cruciate ligament reconstruction model, 1×10^6 BMSCs in 0.35 mL of fibrin glue was injected into bone tunnel as Fibrin-BMSC group. Only fibrin glue (Fibrin group) was injected as control. Three chondrogenesis genes and proteins, including Sox 9, collagen Type II (COII), aggrecan, and three osteogenesis genes and proteins, including Runx2, collagen type I (COI), and osteocalcin, between Fibrin-BMSC and Fibrin group were compared by real-time polymerase chain reaction assay and immunohistochemical assay postoperation. *Results:* In real-time polymerase chain reaction assay, Sox9, COII, aggrecan, COI, and osteocalcin expressions upregulated and Runx2 downregulated were determined in Fibrin-BMSC group at 1 week. COII, aggrecan upregulated, and Runx2 and osteocalcin downregulated were determined at 4 weeks. In immunohistochemical assay, only Sox9, COII, and aggrecan proteins in only Fibrin-BMSC group were observed at 4 weeks. The protein expression as same as gene expression was obtained in a bone tunnel. *Conclusion:* Auto-BMSCs promoted COII and aggrecan expression and reduced Runx2 and osteocalcin expression in a bone tunnel. It demonstrated that these cells could enhance fibrocartilage formation because of higher chondrogenesis expression during tendon-bone healing.

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1. Introduction

Successful anterior cruciate ligament (ACL) reconstruction with a tendon graft requires good healing in tendon-bone junction of the femur and tibia.^{7,13,19} The unstable healing between the tendon and bone results in failed ACL reconstruction.^{26,29} Therefore, the most important thing of tendon-bone healing is a normal-like enthesis structure formation between tendon and bone. For better and quicker tendon-bone healing, many materials have been used to augment or enhance the tendon-bone healing process, such as bone morphogenetic protein-2 (BMP-2), transforming growth factor, fibroblast growth factor (FGF), bone marrow mesenchymal stem cells (BMSCs), calcium phosphate, mesenchymal stem cells (MSCs), BMP-2 gene transfer, synovium-derived stem cell, and the periosteum.^{4,14–16,24,28,30–32,35,37}

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BMSCs were found in 1987. It is a nonhematopoietic cell that exists in bone marrow and owns a potential of multidifferentiation, including differentiation into osteoblast, chondrocyte, fibroblast, adipocyte, and myocyte.¹² Therefore, BMSCs can differentiate into various cells and regenerate new tissue during defects repairing by environment regulations.^{9,10} When BMSCs were implanted into a bone tunnel for ACL reconstruction, they promoted fibrocartilaginous enthesis structure formation and enhanced biomechanical strength between a tendon-bone junction as those between normal ACL and bone.^{22,34}

The transformation of BMSCs during tendon-bone healing is not well understood. Sox9 is expressed in all chondroprogenitors and chondrocytes,^{2,3,25,38} and Runx2 has an essential role in the differentiation of mesenchymal progenitor into osteoblasts.^{11,20,27} To know the gene and protein expressions in an ACL reconstruction in rabbit model, we hypothesize that the fibrocartilage formation between tendon-bone junctions because of BMSCs could express higher chondrogenesis than osteogenesis during early tendon-bone healing. The gene and protein expressed at tendon-bone interface will be checked.

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2. Materials and methods

2.1. Isolation of BMSCs

Around 10 mL of bone marrow was obtained by puncturing into the iliac bone of mature New Zealand white rabbits (mean weight. 3.5 kg). The harvested bone marrow was placed in a 15 mL tube containing 5000 U heparin and the tube was shaken thoroughly to mix the contents. The sample was centrifuged at 1200 revolutions/ min for 5 minutes, and the top liquid was removed and 0.17M NH₃Cl/0.16M tris base in five times the volume of bottom liquid was added. It was mixed completely and allowed to stay at room temperature for 5 minutes for red blood cells to break. The sample was centrifuged at 1200 rpm for 5 minutes. The top liquid was removed and 2% serum/phosphate-buffered saline (PBS) in five times the volume of bottom liquid was added and completely mixed for neutralization. Then, the sample was centrifuged at 1200 rpm for 5 minutes to removed the top liquid and 15 mL of lowglucose Dulbecco's modified Eagle's medium (Gibco BRL Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (HyClone, South Logan, UT, USA) and 1% penicillinstreptomycin (Biological Industries, Kibbutz Beit Haemek, Israel) was added. The cell solution was finely plated in a T75 culture flask (Corning Incorporated, Acton, MA, USA), and the flask was placed into an incubator at 37°C and 5% CO2. Nonadherent cells were removed by changing the medium after 1 day. The culture medium was replenished every 3 days.³⁹ BMSCs can express CD105(+), CD45(+), CD14(-), and CD34(-). The BMSCs were labeled with CD105, CD45, CD14, and CD34 antibody (Dako, Carpinteria, CA, USA) and identified with a flow cytometer (BD FACSCalibur, Franklin Lakes, NJ, USA). The CD105 and CD45 expression was more than 90% and CD14, CD34 expression was less than 15% in our BMSCs.

2.2. Animal study design and surgery

Eighteen mature male New Zealand white rabbits (mean weight, 3.5 kg) were used in this study. Animal treatment conformed to the Guidelines for Care and Use of Laboratory Animals and was approved by the Committee of Experimental Animal Sciences. The rabbits were housed in individual cages and provided with water and commercial rabbit food. The rabbits were divided into two groups: (1) fibrin without cell as positive control (Fibrin group); (2) Fibrin with BMSCs (Fibrin-BMSC group). The fibrin was prepared with 50 U/mL thrombin (Sigma-Aldrich, St Louis, MO, USA) and 12.5 mg/mL fibrinogen (Sigma-Aldrich) at equal volume.

Bilateral ACL reconstructions using the long digital extensor tendon grafts were performed on mature rabbits. Using an aseptic approach, the knee joint was accessed via a lateral parapatellar incision. The long digital extensor tendon was identified and then detached from its insertion on the lateral femoral condyle by sharp dissection. The native ACL was resected. The free tendon was sutured using 3-0 Vicryl suture (Ethicon, Somerville, NJ, USA). Femoral and tibial tunnel with 4 mm diameter were created according to the ACL footprints. The graft was advanced through the tunnel and fixed tightly with 3-0 Nylon. Then, 1×10^6 autologous BMSCs in 0.35 mL of fibrin glue was injected into bone tunnel of left leg in Fibrin-BMSC group.^{23,40} In the Fibrin group, the right leg was treated in the same method but only fibrin glue was injected into bone tunnel. The wounds were closed and the rabbits were allowed to recover in the cages during the experiment period.

Twelve animals were sacrificed with an overdose of pentobarbital at 1 week and 4 weeks for real-time polymerase chain reaction (PCR) assay. Six animals were harvested for histological examination at 4 weeks. Muscles in all limbs were shaved off to observe the position of bone tunnel easily. Bone tunnel tissue was harvested by a 4 mm trephine, as shown in Fig. 1. Then, tendon tissue was discarded and the tissue around the tendon was taken for real-time PCR assay.

2.3. Quantitative real-time PCR assay

Quantification of gene transcripts was determined by quantitative real-time PCR method. Around 0.75 mm thickness tissue (Fig. 1) of tendon-bone junction was harvested and total RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA extracted from gonad of the representative fish was reverse transcribed to the first-strand cDNA using Superscript III with the oligo (dT) primers. The first-strand cDNA was used for the quantitative real-time PCR analysis. According to previous studies, ^{1,6,8,21,23,33,38} quantitative real-time PCR analyses of gene transcripts of target gene were designed for the quantitative real-time PCR, including Sox 9 (chondrogenesis gene). Type II collagen (COII, chondrogenesis gene), aggrecan (chondrogenesis gene), Runx2 (osteogenesis gene), Type I collagen (COI; osteogenesis gene), and osteocalcin (osteogenesis gene). Quantification of standards, samples, and controls were conducted simultaneously by real-time PCR (GeneAmp 5700 Sequence Detection System; Applied Biosystems, Foster city, CA, USA) with SYBR green I as a dsDNA minor-groove binding dye. The standard curves of log (transcript concentrations) versus C_T (the calculated fractional cycle number at which the PCR-fluorescence



Fig. 1. Surgery design of anterior cruciate ligament reconstruction in tendon-bone junction.

product is detectable above a threshold) were obtained. The correlation of the standard curve was -0.999. The values detected from different amounts of RNA (10-fold dilution series) of the representative samples aligned in parallel with the respective standard curve. The transcript values of each gene were calibrated with internal control *gapdh* and then normalized.

2.4. Histological examination

Six rabbits (six limbs each group) were prepared for histological and immunohistochemical evaluation at 4 weeks. The femur of rabbits was harvested. The specimen was fixed with 10% formalin for 24 hours. After decalcification and dehydration in graded alcohols, they were then embedded in paraffin, sectioned to a thickness of 5 μ m, and processed for hematoxylin and eosin staining. Histological sections were observed and photographed by a light microscope (1X70; Olympus, Tokyo, Japan). Ten sections were selected randomly to calculate average thickness of new bone, and three most thickness positions of new bone were determined in every section.

2.5. Immunohistochemistry assay

Tissue sections were deparaffinized, treated with 3% hydrogen peroxide in PBS for 20 minutes to inhibit endogenous peroxidation, rinsed with PBS containing surfactant Tween-20 (Sigma-Aldrich), and blocked for 30 minutes in PBS containing 5% goat serum. To assess protein expression, the sections were conjugated with primary antibodies for 1 hour, including polyclonal antibody to anti-Sox 9 (Abcam, Cambridge, United Kingdom; 1:500 dilution), COII (Calbiochem, Darmstadt, Germary; 1:200 dilution), and aggrecan (Novus Biologicals, Littleton, CO, USA, 1:500 dilution) to assay chondrogenesis expressions, and Runx2 (Abcam; 1:500 dilution), COI (Abcam; 1:100 dilution), and osteocalcin (Abcam; 1:500 dilution) to assay osteogenesis expressions. Then, primary antibodies were removed from the sections, secondary antibody was added onto the samples and allowed to stand for 30 minutes. It was then washed three times with PBS, incubated in DAB (Dako, Glostrup, Denmark) for 40 seconds, and the nucleus was stained with hematoxylin. All sections were observed under a light microscope. The rabbit articulus tissue (including sponge bone and cartilage) is used as negative and positive control for above immunohistochemistry assay (data not shown).

2.6. Statistical analysis

All statistical calculations were carried out using Student *t* test. Means (\pm standard deviations) were reported as appropriate. Values of *p* < 0.05 were considered statistically significant.

3. Results

3.1. Real-time PCR

Specimens of Fibrin and Fibrin-BMSC groups were analyzed at 1 week and 4 weeks postoperatively. Upregulated gene expressions of Sox9, COII, aggrecan, COI, and osteocalcin and downregulated Runx2 were determined comparing those in Fibrin-BMSC group with those in Fibrin group at 1 week (Fig. 2A). Upregulated COII and aggrecan and downregulated Runx2 and osteocalcin were obtained comparing those in Fibrin-BMSC group with those in Fibrin group at 4 weeks (Fig. 2A). In Fibrin-BMSC group, chondrogenesis and osteogenesis were found in early healing. In Fibrin group (without BMSCs), only higher osteogenesis was obtained during healing (Fig. 2).

3.2. Histology and immunohistochemistry assay

3.2.1. Hematoxylin-eosin staining

Cross-sections of the bone tunnel showed that there was little new bone, and no fibrocartilage structure formed at 4 weeks in both groups (Fig. 3). Fibrin (Fig. 3B) and Fibrin-BMSC (Fig. 3D) groups had $23 \pm 12 \,\mu\text{m}$ and $62 \pm 9 \,\mu\text{m}$ of new bone thickness, respectively. The more thickness of new bone tissue around tendon in Fibrin-BMSC group (Fig. 3B and D) than those in Fibrin group (Fig. 3A and C) was found. There were unmatured bone tissue formations in bone marrow area near the new bone (Fig. 3D).

3.2.2. Sox9 staining

Immunohistochemistry for Sox9 showed protein expression from chondrocytes. It was an indication that BMSCs differentiated into chondrocytes. No Sox9 expression in Fibrin group was found (Fig. 4A and C). Strong Sox9 expression near new bone in Fibrin-BMSC group was observed at 4 weeks (Fig. 4B and D). This result indicated that fibrocartilage-like tissue could start to form during early tendon-bone healing in Fibrin-BMSC group but not in Fibrin group (Fig. 4).

3.2.3. COII staining

Immunohistochemistry for COII showed protein expression from chondrocytes. It was an indication that BMSCs differentiated to chondrocytes. A little COII expression in Fibrin group was found (Fig. 5A and C). Strong COII strong expression near new bone in Fibrin-BMSC group was observed (Fig. 5B and D). COII expression also indicated that fibrocartilage-like tissue may form during early tendon-bone healing in Fibrin-BMSC group (Fig. 5).



Fig. 2. Gene expression ratio after surgery for (A) 1 week and (B) 4 weeks. *Significant difference between Fibrin and Fibrin-BMSC group, p < 0.05. BMSC = bone marrow mesenchymal stem cell; COII = type II collagen.



Fig. 3. Histology of tendon-bone healing tunnel after 4 weeks, hematoxylin and eosin stained. (A) Fibrin (magnified at \times 40); (B) Fibrin-bone marrow mesenchymal stem cell (BMSC) (magnified at \times 40); (C) Fibrin (magnified at \times 200); and (D) Fibrin-BMSC (magnified at \times 200). BM = bone marrow; NB = new bone; T = tendon.

3.2.4. Aggrecan staining

Immunohistochemistry for aggrecan also showed glycosaminoglycan expression from hyaline chondrocytes. It was also an indication that BMSCs differentiated to chondrocytes. Fewer expression of aggrecan in Fibrin group was found (Fig. 6A and C). Strong aggrecan strong expression near new bone in Fibrin-BMSC group was observed (Fig. 6B and D). Aggrecan expression indicated that fibrocartilage-like tissue may form in Fibrin-BMSC group (Fig. 6). This data indicated that Fibrin-BMSC group could enhance cartilage-like tissue formation.



Fig. 4. Immunohistochemistry assay of Sox9 in tendon-bone healing tunnel after surgery for 4 weeks. (A) Fibrin (magnified at \times 40); (B) Fibrin-bone marrow mesenchymal stem cell (BMSC) (magnified at \times 40); (C) Fibrin (magnified at \times 200); and (D) Fibrin-BMSC (magnified at \times 200). Black arrow = Sox9; BM = bone marrow; NB = new bone; T = tendon.



Fig. 5. Immunohistochemistry assay of collagen Type II in tendon-bone healing tunnel after surgery for 4 weeks. (A) Fibrin (magnified at \times 40); (B) Fibrin-bone marrow mesenchymal stem cell (BMSC) (magnified at \times 40); (C) Fibrin (magnified at \times 200); and (D) Fibrin-BMSC (magnified at \times 200). Black arrow = collagen type II; BM = bone marrow; NB = new bone; T = tendon.

3.2.5. Runx2 staining

Immunohistochemistry for Runx2 indicated protein expression from osteoblasts. It was an indication that BMSCs differentiated to osteoblasts. Runx2 expression was mainly found near the new bone and tendon in Fibrin group (Fig. 7A and C). A little of Runx2 expression near new bone in Fibrin-BMSC group was observed (Fig. 7B and D). Runx2 expression also indicated osteogenesis during early tendon-bone healing in Fibrin group (Fig. 7).

3.2.6. COI staining

Immunohistochemistry for COI indicated protein expression from tenocytes or osteoblasts. It was one of the indications that



Fig. 6. Immunohistochemistry assay of aggrecan in tendon-bone healing tunnel after surgery for 4 weeks. (A) Fibrin (magnified at ×40); (B) Fibrin-bone marrow mesenchymal stem cell (BMSC) (magnified at ×40); (C) Fibrin (magnified at ×200); and (D) Fibrin-BMSC (magnified at ×200). Black arrow = aggrecan; BM = bone marrow; NB = new bone; T = tendon.



Fig. 7. Immunohistochemistry assay of Runx2 in tendon-bone healing tunnel after surgery for 4 weeks. (A) Fibrin (magnified at ×40); (B) Fibrin-bone marrow mesenchymal stem cell (BMSC) (magnified at ×40); (C) Fibrin (magnified at ×200); and (D) Fibrin-BMSC (magnified at ×200). Black arrow = Runx2; BM = bone marrow; NB = new bone; T = tendon.

BMSCs differentiated to osteoblasts. COI expression in new bone and tendon were observed in both groups (Fig. 8). COI expression also indicated osteogenesis during early tendon-bone healing in both groups (Fig. 8).

3.2.7. Osteocalcin staining

Immunohistochemistry for osteocalcin was one of the indicators for osteogenesis. It was one of indication that BMSCs differentiated to

osteoblasts. Osteocalcin expression at the new bone in both groups was observed (Fig. 9). More osteocalcin expression near the new bone in Fibrin group was found (Fig. 9A and C). A little of osteocalcin expression near the new bone in Fibrin-BMSC group was observed (Fig. 9B and D). Osteocalcin expression also indicated osteogenesis during early tendon-bone healing in Fibrin group (Fig. 9).

All gene and protein expressions in tendon-bone junction at 4 weeks were concluded in Table 1. In both groups, the result of



Fig. 8. Immunohistochemistry assay of collagen Type I in tendon-bone healing tunnel after surgery for 4 weeks. (A) Fibrin (magnified at \times 40); (B) Fibrin-bone marrow mesenchymal stem cell (BMSC) (magnified at \times 40); (C) Fibrin (magnified at \times 200); and (D) Fibrin-BMSC (magnified at \times 200). Black arrow = collagen type I; BM = bone marrow; NB = new bone; T = tendon.

Fig. 9. Immunohistochemistry assay of osteocalcin in tendon-bone healing tunnel after surgery for 4 weeks. (A) Fibrin (magnified at \times 40); (B) Fibrin-bone marrow mesenchymal stem cell (BMSC) (magnified at \times 40); (C) Fibrin (magnified at \times 200); and (D) Fibrin-BMSC (magnified at \times 200). Black arrow = osteocalcin; BM = bone marrow; NB = new bone; T = tendon.

gene expressions was the same as proteins expressions at 4 weeks. In Fibrin-BMSC group, higher chondrogenesis and lower osteogenesis were found in early healing. In Fibrin group, higher osteogenesis was obtained during healing.

4. Discussion

Better tendon-bone healing in ACL reconstruction using tendon grafts depends on biological integration between the tendon and bone. Fibrous integration of the tendon graft to the bone was observed immediately after tendon transfer and following remodeling of the bone tunnel. FGF and vascular growth factors were found in abundance at the tendon-bone interface in the first 3 weeks of graft incorporation. The early tendon-bone healing may be important in controlling the integration process of the interface in ACL reconstruction surgery as seen in a rabbit model.¹⁹ In previous studies, graft tissues have no contribution to repair of the tendon-bone interface, many materials, such as BMP-2, transforming growth factor, FGF, BMSC, calcium phosphate, MSC, BMP-2 gene transfer, and the periosteum, have been used to augment or enhance the tendon-bone healing

Table 1	
Gene and protein expressions in tendon-bone junction at 4 weeks.	

Group	Fibrin		Fibrin-BM	SC
Expression	Gene	Protein	Gene	Protein
Sox9	0	_	0	+
Collagen Type II	_	_	+	+
Aggrecan	_	-	+	+
Runx2	+	+	_	_
Collagen Type I	0	+	0	+
Osteocalcin	+	+	_	_

+: Gene upregulated and higher protein expression.

-: Gene downregulated and lower protein expression.

O: The same gene expression.

BMSC = bone marrow mesenchymal stem cell.

process. Previous study had showed that when ACL reconstruction with the tendon wrapped with periosteum, the periosteum could form the fibrocartilage at the interface of tendon graft and bone tunnel with its progenitor cell in rabbit model.^{6,7,28} With BMSCs, it also could stimulate new bone ingrowths and fibrocartilage formation.^{22,34}

BMSCs could differentiate into osteoblasts and chondrocytes. Specific transcription factors regulate the differentiation pathways of chondrocytes and osteoblasts. Sox9, a transcription factor with a high-mobility group DNA-binding domain, activates chondrocytespecific marker genes, such as COII and aggrecan.^{5,21,33} Sox9 is expressed in all chondroprogenitors and chondrocytes except hypertrophic chondrocytes.^{2,3,25,38} Moreover, inactivation of Sox9 during or after mesenchymal condensations results in a very severe chondrodysplasia, which is characterized by an almost complete absence of cartilage in the endochondral skeleton.¹ Runx2 is a member of the Runt-domain family of transcription factors, which form heterodimers with a single ubiquitous polypeptide called Cbfb.³⁶ In addition to the Runx DNA binding domain, Runx2 contains an active transactivation domain, rich in glutamine and alanine residues, and activates the osteocalcin and COI genes.^{11,17} Runx2 has an essential role in the differentiation of mesenchymal progenitor into osteoblasts.^{11,20,27} Therefore, we analyzed Sox 9, COII, aggrecan, Runx2, COI, and osteocalcin gene and protein expressions to observe the transformation of BMSCs during tendon-bone healing.

In this study, BMSCs exhibited more collagen deposition and increased proliferation of cartilage-like cells, which was indicated by positive COII immunostaining of the tendon-bone interface at 4 weeks. In contrast, treatment without BMSCs demonstrated that progressive maturation and reorganization of fibrous tissue aligned along the load axis. In previous studies, when introducing BMSCs to the bone tunnel, the insertion healing of tendon to bone will be improved by fibrocartilagenous attachment formation.^{28,34}

To realize the effect of BMSCs on fibrocartilage formation during tendon graft-to-bone healing, not only protein but also the gene expressions are determined. And the gene expression of chondrocytes were upregulated, the gene expression of osteoblasts were downregulated after BMSCs implantation at 1 week. The same tendency of protein expressions was observed at 4 weeks. Therefore, fibrocartilage formation may easily stimulated by auto-BMSCs implantation. In this study, auto-BMSCs express higher chondrogenic-related genes (Sox9, COII, and aggrecan) and lower osteogenic-related genes (Rux2, COI, and osteocalcin) expression. These data showed that delayed osteoblasts proliferations/differentiation but stimulated fibrocartilage formation when enhancing tendon-bone healing with BMSCs. BMSCs synthesize and secrete specific cytokines and growth factors, and the induction into each differentiation pathway involves modulation of their production, as well as regulation of particular signal-transduction pathway proteins.

According to the real-time PCR results, BMSCs transplanted into bone tunnel (Fibrin-BMSC group) was compared with Fibrin group (without BMSCs treatment) at 1 week. The strong chondrogenesis expressions and weak osteogenesis expressions in Fibrin-BMSC group were found. It was in contrast to the findings in Fibrin group. At 4 weeks, the Fibrin-BMSC group not only showed strong chondrogenesis expressions but also showed strong osteogenesis expressions. And the Fibrin group always showed higher osteogenesis expressions at 1 week and 4 weeks. In summary, the present study demonstrates the important role of BMSCs by delayed osteoblasts proliferations/differentiation but stimulated fibrocartilage formation when enhancing tendon-bone healing. The involvement and association of BMSCs in regulating microenvironment of bone tunnel as well as being a pluripotent cells source are also demonstrated.

5. Conclusion

In this study, Fibrin-BMSC group promoted COII and aggrecan expression, and reduced Runx2 and osteocalcin expression was demonstrated. For tendon-bone healing in ACL reconstruction by auto-BMSCs implanted into a bone tunnel, fibrocartilage could be easily formatted with time after operation was concluded.

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