Tendon–bone interface healing using an injectable rhBMP-2-containing collagen gel in a rabbit extra-articular bone tunnel model

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Abstract

This study examines the hypothesis that injectable collagen gel can be an effective carrier for recombinant human bone morphogenetic protein-2 (rhBMP-2)'s localization to the healing tendon–bone interface. In 36 mature New Zealand White rabbits, the upper long digital extensor tendon was cut and inserted into the proximal tibial bone tunnel. Then a rhBMP-2-containing collagen gel was injected into the tendon–bone tunnel interface, using a syringe. Histological and biomechanical assessments of the tendon–bone interface were conducted at 3 and 6 weeks after implantation. In vitro testing showed that the semi-viscous collagen gel at room temperature was transformed into a firm gel state at 37°C. The rhBMP-2 release profile showed that rhBMP-2 was released from the collagen gel for more than 28 days. In vivo testing showed that fibrocartilage and new bone are formed at the interface at 6 weeks after injection of rhBMP-2. On radiography, spotty calcification appeared and enthesis-like tissue was produced successfully in the tendon at 6 weeks after injection of rhBMP-2. Use of the viscous collagen gel and rhBMP-2 mixture increased the fusion rate between the bone tunnel and tissue graft. This study demonstrates that viscous collagen gel can be an effective carrier for rhBMP-2 delivery into surgical sites, and that the injectable rhBMP-2-containing collagen gel may be applied for the enhancement of tendon–bone interface healing in the future. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords rhBMP-2; tendon; bone tunnel; enthesis; ligament injury

1. Introduction

Rupture of tendon and ligament tissue is one of the most common sports injuries (Miyasaka et al., 1991; Woo et al., 2006). To reconstruct ruptured tissues, types of tendon graft, such as semitendinosus, gracilis, tibialis and peroneous longus tendons, are transplanted. Although the use of tendon grafts has become popular in reconstruction surgery, the outcomes after surgery are often poor because of their poor healing capacity (Kuo et al., 2010). Moreover, high stress may accumulate at the interface, due to the difference in mechanical properties of the two materials. The regeneration of a unique transitional tissue called an ‘enthesis’ may effectively transfer the stress from tendon to bone tissue (Lui et al., 2010). For successful tendon and ligament reconstruction, osteointegration of tendon grafts with the patient’s bone tissue is essential.

Recombinant human bone morphogenetic proteins (rhBMPs) are physiological agents responsible for the inherent potential of bone to regenerate. They promote the differentiation of early-stage mesenchymal cells into chondrogenic and osteogenic lineages that support new bone formation (Lee et al., 2011). The bone–tendon junction is similar to endochondral ossification, and recapitulation of this process in the tendon or ligament might induce formation of an enthesis postnatally (Hashimoto et al., 2011). rhBMP-2 delivery systems based on various materials, such as collagen gels and sponges, for the prolonged and local release of rhBMP-2, have been studied to significantly enhance new bone formation (Lee et al., 2012).
In previous studies, rhBMP-2 was delivered to the repair site of the bone–tendon interface using a collagen sponge (Rodeo et al., 1999; Thomopoulos et al., 2012). However, it was necessary to develop delivery systems that localized the BMP-2 to the healing point and minimized leakage from the tunnel. Further studies about alternative delivery systems are required for BMP-2.

For the effective delivery of rhBMP-2 into surgical sites, an injectable material, such as viscous collagen gel, can be used. Moon et al. (2009) reported a comparative study of the osteogenic effectiveness of rhBMP-2 between collagen sponge and collagen gel in a rat spinal fusion model. They pointed out that the method of introducing rhBMP-2 in a collagen sponge had provoked the problems of leakage of BMP. Collagen gel is FDA-approved and has been widely used clinically (Malafaya et al., 2007). The resorption time of most commercially available collagens is approximately 6 months to 1 year (Premaraj et al., 2006). This material allows clinicians to optimize the delivery of rhBMP-2 for consistent release over a desired time period.

We hypothesized that viscous collagen gel could be an effective carrier for localization of BMP to the tendon–bone interface during the early regeneration period after reconstruction surgery.

2. Materials and methods

2.1. Preparation of rhBMP-2

Briefly, rhBMP-2 is a disulphide-linked dimeric protein molecule with two major subunit species of 114 and 131 amino acids. Each subunit is glycosylated at one site with high-mannose-type glycans. rhBMP-2 is produced by a genetically engineered Chinese hamster ovary cell line. The final purified rhBMP-2 solution used in this study (Cellumed Co. Ltd, Seoul, Korea) was reconstituted and diluted in distilled water to a concentration of 50 μg/ml.

2.2. Conjugation of collagen gel and rhBMP-2

Collagen gel (1%) from porcine skin (Matrixen-PSC; Bioland, Cheonan, Korea) was mixed with 50 μg/ml rhBMP-2. Previous studies using rhBMP-2 made by Cellumed showed that the concentration of 50 μg/ml rhBMP-2 affected osteogenic effectiveness in both rat and rabbit models (Moon et al., 2009; Kim et al., 2012). The same concentration of rhBMP-2 was applied in the present study. In order to confirm the temperature dependence of the collagen sol–gel phase transition, the optical density of both 100 μl and 200 μl 1% collagen gel at 37°C was determined at 313 nm, using an absorbance microplate reader (cat. no. 1420, Perkin-Elmer, Miami, FL, USA) at 10, 20 and 30 min.

The conjugated rhBMP-2 gel was placed into a 12-well plate containing 1 ml phosphate-buffered saline, pH 7.4, for checking the release phase, and incubated at 37°C. The in vitro release of rhBMP-2 occurred over a period of 28 days and a cumulative release curve was plotted. At each time point (1, 3, 5, 7, 14 and 28 days), each supernatant was harvested. Then, the rhBMP-2 release quantity was analysed using an enzyme-linked immunosorbent assay (ELISA) kit (cat. no. DBP200, R&D Systems, Minneapolis, MN, USA).

2.3. Animal study design and operative procedure

Thirty-six healthy adult New Zealand White rabbits weighing 3.0–3.5 kg were used in this study, aimed at generating a new enthesis at the interface between the tendon and tibial bone tunnel. Animal treatment conformed to the Guidelines for Care and Use of Laboratory Animals and was approved by the Committee of Experimental Animal Sciences. All the rabbits were equally divided into three groups: saline only (control); collagen gel without rhBMP-2; and rhBMP-2-conjugated collagen gel. The animals were randomly assigned and euthanized at 3 and 6 weeks. Ketamine 40 mg/kg with xylazine 5 mg/kg (Rompun, Bayer Healthcare, Leverkusen, Germany) was injected intramuscularly to induce general anaesthesia. The rabbits underwent an operative procedure for an extra-articular tendon–bone healing model, in which no mechanical loading occurred at the rerouted long digital extensor tendon (Kim et al., 2002). With the use of an aseptic approach, the knee joint was accessed through a lateral parapatellar incision. The long digital extensor tendon was identified and then detached, by sharp dissection, from its insertion at the lateral femoral condyle. The free tendon was sutured with 3–0 vicryl sutures (Ethicon, Somerville, NJ, USA). Then, the fascia covering the anterior tibial muscle was incised, and the muscle was retracted laterally. A bone tunnel was created in the proximal tibial metaphysis at a 30° angle relative to the long-bone axis, using a drill of 2 mm diameter. The average size of tunnel was 2.09 ± 0.04 mm diameter and 5.13 ± 0.05 mm length, which were measured by randomly selected micro-computed tomography (micro-CT) images after animal study. The free end of the tendon was pulled manually through the drill hole and sutured to the periostium and soft tissue at the medial aspect of the proximal tibia, using 3–0 nylon sutures (Figure 1).

A 200 μl aliquot of rhBMP-2 (10 μg)-conjugated collagen gel was injected into the tendon–bone junction. The contralateral limb received a similar operation. The joint capsule, fascia and subcutaneous tissue were closed with interrupted 3–0 vicryl sutures and the skin was closed with interrupted 3–0 nylon sutures.

2.4. Analysis of three-dimensional (3D) CT and bone mineral density

A micro-CT system (Skyscan X-ray Microtomography 1173; Skyscan, Kontich, Belgium) was used to quantify the bone mineral density (BMD) and mineralized tissue density (BMD) in the bone mineral density (BMD) and mineralized tissue.
Tendon–bone fusion using injectable rhBMP-2-containing collagen gel

ingrowth inside the tendon–bone tunnel. Specimens were scanned perpendicular to the long-bone axis covering the entry and exit of the bone tunnel. The sections were reconstructed using 3D reconstruction bundle software (ANT-software, Skyscan, Kontich, Belgium). To quantify the amount of newly formed mineralized tissue over time, a 3 mm circular region of interest (ROI) inside the tendon–bone tunnel was chosen and reconstructed using the 3D software. After thresholding, the BMD (mg/cm²) of the mineralized tissue inside the tendon–bone interface was calculated.

2.5. Biomechanical testing

Knee joint tissues, including the tendon–bone tunnel site, were harvested and stored at −20°C until biomechanical testing. After the tissues had been thawed overnight at room temperature, the knee joint was carefully dissected to remove the surrounding soft tissues. To analyse the physical properties, tensile strength was measured using a Universal Testing Machine (DUS-200, ORIENTAL TM, Siheung, Korea). The specimen was held vertically on a 5000 N load cell, and tensile strength was measured by pulling the sample at a load displacement rate of 10 mm/min tensile force (Hashimoto et al., 2007), then the failure load and ultimate strength (N) were recorded.

2.6. Histological and histomorphometric analyses

After the rabbit knee tissues had been dissected to remove all the soft tissues, the specimens were fixed in a

<table>
<thead>
<tr>
<th>Characteristic</th>
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<td>Fibrocartilage formation</td>
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<td>Abundant</td>
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<td>New bone formation</td>
<td></td>
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<tr>
<td>Abundant</td>
<td>3</td>
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<td>Moderate</td>
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<td>Slight</td>
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<td>None</td>
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<tr>
<td>Tendon graft bonding to adjacent tissue</td>
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<tr>
<td>75–100%</td>
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<td>50–75%</td>
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Figure 1. Operative procedure of the long digital extensor tendon sutured to the periosteum and soft tissue of rabbit medial tibia

Table 1. Histomorphometric analysis to assess healing of the tendon within the bone tunnel (full score = 9 points)

Figure 2. Ultraviolet turbidity of 1% collagen gel at each time point, dependent on temperature; average of triplicates at each time point which is 0, 10, 20 and 30 min at 37°C incubation
neutralized formalin solution for 2 days and decalcified using 10% formic acid. The specimens were then dehydrated in ethanol and embedded in paraffin. Subsequently, the specimens were sliced into 4 μm-thick sections in an orientation parallel to the bone tunnels, and each section was mounted on a glass slide and dried at 60°C for 2 h. The sections were stained with Masson’s trichrome and visualized using an optical microscope. Healing of the tendon–bone interface was graded histomorphologically by two blinded observers, according to a published protocol (Yeh et al., 2007).

**Histomorphometric analysis** was performed to assess healing of the bone–tendon interface. Quantitative histomorphometric analysis was done by two blinded observers, who apportioned 0–3 points, based on three individual histomorphological criteria: fibrocartilage formation; new bone formation; and tendon graft bonding to adjacent tissue (Table 1).

### 2.7. Statistical analysis

The data were averaged from at least triplicate samples. The same experiments were repeated three times to ensure the reproducibility of the methods used. All statistical analyses were performed using commercially available software (SPSS v. 15.0; IBM Corp., Armonk, NY, USA). The post hoc Scheffé test was used to analyse differences between groups, with significance levels set at * p < 0.05 and ** p < 0.01.

### 3. Results

Turbidity of the collagen gel at 37°C was greatly increased between the time points of 10 (OD 0.953) and 20 (OD 4.099) min. At the time point of 30 (OD 4.451) min, the viscous collagen sol was transformed into the gel state, which was not flowing (Figure 2).

The release of rhBMP-2 was maintained for > 28 days: 50% of the total quantity of rhBMP-2 was released from the collagen gel within 5 days after incubation, while the other half was released slowly for over 28 days, by which time 89.3% of the total rhBMP-2 had been released. The condition of the rhBMP-2-collagen gel mixture showed a slow-release phase of the rhBMP-2 (Figure 3).

According to 3D CT, the distal epiphyseal plate of rabbits had limited cancellous bone. Nevertheless, new bone formation was detected at the interface between the tendon and tibial bone tunnel in the rhBMP-2+ group after 3 weeks; the control and rhBMP-2/C0 groups did not show new bone formation (Figure 4). After 6 weeks, the rhBMP-2+ group showed higher new bone formation compared with the control and rhBMP-2/C0 groups. In addition, the BMD of the rhBMP-2+ group was significantly higher than that of the control group after 3 and 6 weeks.

**Figure 3.** Release profile of rhBMP-2 from 1% collagen solution; average of triplicates at each time point for 4 weeks

**Figure 4.** 3D CT images of the enthesis generated by transfer of the toe flexor or rhBMP-2+ or rhBMP-2/C0 bone complex to the proximal tibia at 3 and 6 weeks; w, weeks
(Figure 5). The BMD of the rhBMP-2− group was slightly higher than that of the control group, but was not significantly different.

In biomechanical testing, the ultimate failure load of the rhBMP-2+ group was higher than that of the control and rhBMP-2− groups at 3 and 6 weeks (Figure 6). After 3 weeks, the ultimate failure load of the rhBMP-2+ group was 2.5-fold higher than that of the control group; after 6 weeks, it was 1.8-fold higher. However, there was no significant difference between the rhBMP-2− and control groups.

After 3 weeks, Masson's trichrome staining showed that collagen fibres and fibrous cartilage were widely detected in the implanted tendons of the rhBMP-2+ group. The new bone was partly between the tendon and host bone (Figure 7). After 6 weeks, there was increased fibrous cartilage and new bone between the tendon and host bone, and new Sharpey-like fibres were detected in the rhBMP-2+ group (Figure 8).

In histomorphometric analysis, the histological score for the enthesis of the rhBMP-2+ group was significantly higher than that of the control and rhBMP-2− groups (Figure 9). Moreover, the results after 6 weeks were higher than those after 3 weeks in the rhBMP-2+ group.

4. Discussion

During the healing process after tendon and ligament reconstruction surgery, stable enthesis generation at the interface between the tendon and bone tunnel is one of the most important conditions. rhBMP-2 can be used as the growth factor because of its role in inducing differentiation of osteoprogenitor cells to osteoblasts (Takada et al., 2003). For effective soft tissue healing, rhBMP-2 application may be the best method for new bone formation between the tendon and bone tunnel. However, rhBMP-2...
requires a scaffold for embedding (Luca et al., 2010; Tsujigiwa et al., 2005). It is important to develop a rhBMP-2 delivery system for its immobilization. The immobilization of rhBMP-2 can improve the host cell interaction between the implant and host tissue, thereby stimulating cellular activity (Mooney et al., 2005; Li and Wozne, 2001).

We used visco-elastic collagen gel for the minimum loss and steady release of rhBMP-2. Collagen gels are an injectable and biocompatible drug delivery matrix, which suggests sustained release of therapeutic molecules. Collagen gels have also been employed as scaffolds in tissue engineering (Donald and Joel, 2003). When the collagen gel is injected into the implantation site, it is easy to use due to its viscous solution state. After implantation, it becomes semi-solid at body temperature, and this thermo-sensitive state can establish stable implantation during soft tissue-grafting surgery.

In this study, a rabbit model was used to investigate the healing of soft tissue grafts within the bone tunnels. We developed an improved injectable gel filling system for tendon-bone interface healing using viscous collagen gel and rhBMP-2.

In vitro testing showed that the gel state of the collagen gel at room temperature was transformed into the sol state at body temperature (37°C). This finding demonstrates that collagen gel can be effective for the slow and stable release of rhBMP-2 without any loss by irrigation during surgery. This control of new bone formation depends on the rate of collagen gel degradation and the time course of stimulation of osteogenesis (Patel et al., 2006). The conjugation of rhBMP-2 and collagen gel can be implanted in a specific position without other subcutaneous bone formation in unintended areas. Collagen gel resorption occurs over the first 7–14 days after implantation. Therefore, viscous collagen gel could protect the whole diffusion of rhBMP-2 after transformation into the sol state at body temperature.

In vivo testing showed that the use of the collagen gel–rhBMP-2 mixture increased the fusion rate between the bone tunnel and tissue graft. Results of BMD analysis also showed that the quality of new bone formation by rhBMP-2 treatment was better than that in the non-rhBMP-2 groups.

A limitation of this study is that we need to demonstrate the effectiveness of various dose treatments and perform long-term follow-up for a rabbit model. Moreover, a bigger animal model that has rich cortical and cancellous bone would be ideal.
cancellous bone, such as a canine or porcine model, should be investigated; we should design and perform a further study.

In conclusion, injectable rhBMP-2-containing collagen gel induced earlier and better new bone formation at the tendon–bone tunnel interface. The clinical application of injectable rhBMP-2 can be used for the enhancement of soft tissue reconstruction in the future. This study demonstrated that the combination of collagen gel and rhBMP-2 can accelerate the healing process for bone fusion between bone tunnel and tissue graft.

References


Conflict of interest

The authors have declared that there is no conflict of interest.

Acknowledgements

This study was supported by Eulji University, Bio Meditech, RIC study (Grant No. 2012-02-06) and the Ministry of Trade, Industry and Energy (Grant No. 10037842).