Effect of Bone Morphogenetic Protein-2 on Tendon–Bone Integration in an In Vitro Cell Culture

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Abstract

The goal of this study was to evaluate the influence of bone morphogenetic protein-2 (BMP-2) on tendon–bone integration in a bovine in vitro cell culture. Seventy-two bovine tendons were cultivated over 3 months. The effects of BMP-2 were evaluated by generation in 4 subgroups. The groups differed in 2 parameters: the application of BMP-2 and the application of primary bovine osteoblasts. Results were analyzed biochemically by determining alkaline phosphatase activity and histologic tendon calcification, both markers for graft incorporation. Histological analysis demonstrated a positive effect of BMP-2 on the production of extracellular matrix and therefore the induction of osteogenesis. In addition, the results showed a superior cell ingrowth on the tendon in the BMP-2–stimulated groups. Calcium carbonate–like structures and organized ossification zones could only be detected in the BMP-2–stimulated tendons. The histological results matched those of the biochemical alkaline phosphatase analysis. The highest alkaline phosphatase activity was detected using BMP-2 stimulation in the first month (P < .001). High alkaline phosphatase values suggest high osteoblast activity and a high potential for mineralization. Furthermore, a positive effect of BMP-2 on fibroblasts existed with regard to the overall integration process. These results confirm the positive influence and triggering effect of BMP-2 on the mineralization process. Bone morphogenetic protein-2 seems to accelerate and optimize tendon–bone integration in the early process of graft incorporation. Besides the influence of BMP-2 on bovine osteoblasts, an additional positive effect of BMP-2 on bovine fibroblasts was detected; therefore, graft incorporation may be carried out by osteoblasts and fibroblasts.

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Figure: Photographs of histological comparison of groups 1 and 2 after 12 weeks of cultivation (von Kossa stain, original magnification ×10). Photograph from group 1 showing significant amounts of dark brown- and black-stained elements on the surface of the tendon, representing different stages of already calcified parts of the tendon (arrows) (A). Photograph from group 2 showing a small cell layer with a few brown-stained cells on the edge of the tendon (arrow). No calcification is seen (B).
Rupture of the anterior cruciate ligament (ACL) represents one of most common injuries in amateur and professional sports. Currently, more than 100,000 ACL reconstructions are performed annually in the United States. Modern arthroscopic management and reconstruction provide a high degree of patient satisfaction and good clinical results. Individualized postoperative rehabilitation programs are a basic requirement for overall therapeutic success. Nevertheless, complete convalescence might take 6 months or more.

Operative reconstruction is generally accepted to avoid long-term consequences, such as meniscus or cartilage damage and osteoarthritis. However, up to 8% of patients need operative revision because of recurrent instability due to graft failure. Therefore, tendon–bone interface are significant factors in ACL surgery, and successful graft incorporation remains of vital importance for clinical outcome.

Firm attachment, graft remodeling, and graft incorporation at the tendon–bone interface are significant factors in ACL reconstruction. Recent research has focused on the integration processes of grafts. In experimental animal models and with human patients, integration of the fibrovascular interface and bone tunnel remodeling have been shown to contribute to progressive connection between the tendon graft and surrounding bony structures. However, successful graft incorporation of the newly formed tendon insertion must be completed to provide a tensile strength comparable with that of normal ACL insertions.

The success of ACL reconstructive surgery depends on initial graft fixation and subsequent biological integration of the graft to the bone. Based on the current literature, several growth factors, such as bone morphogenetic protein (BMP), are important in this process by acting on cellular components at the tendon–bone interface, thus promoting healing and improving mechanical strength. Bone morphogenetic proteins represent a group of 15 to 30 kDa molecules that belong to the transforming growth factor beta family. Their ability to induce ossification was first reported by Urist in 1965. Bone morphogenetic protein has been shown to be effective in promoting bone formation and tendon–bone healing in a bone tunnel. Rodeo et al reported that the additional application of recombinant human BMP-2 enhances tendon healing in a bone tunnel. Martinek et al reported that adenovirus–luciferase–transfected adenovirus–BMP-2 gene transfer significantly improves the integration of double-bundle semitendinosus tendon grafts in bone tunnels after ACL reconstruction in rabbits.

The goal of the current study was to evaluate the influence of BMP-2 on tendon–bone integration in an in vitro cell culture. The authors hypothesized that additional external application of BMP-2 may provide better and faster graft incorporation in in vitro conditions, focusing on markers for graft incorporation, such as alkaline phosphatase. In addition, histological examinations were performed to evaluate potentially enhanced tendon–bone interactions.

**Materials and Methods**

**Experimental Setup**

In an in vitro cell culture, 72 bovine tendons were cultivated over 3 months. Potential effects of BMP-2 on the process of tendon–bone integration were evaluated by generation in 4 subgroups with 18 specimens in each group. The groups differed in 2 parameters: the additional application of BMP-2 (400 ng/mL) and the application of primary bovine osteoblasts (POBs), which have been previously cultivated for use in this study. Their application was varied according to the following schematic: group 1, tendon + POBs + BMP-2 (n = 18); group 2, tendon + POBs (n = 18); group 3, tendon + BMP-2 (n = 18); group 4, tendon only (n = 18).

Cultivation of the POBs was performed after extraction of the POBs from the periosteum of young bovines. Then, a modified BGJ-medium containing 320,000 POBs/mL was applied to groups 1 and 2. The overall study period was 12 weeks. Quantitative biochemical analysis focusing on alkaline phosphatase (ALP) activity was performed by weekly scheduled measurement of the medium overlap. Furthermore, 6 tendons were extracted from each group for further histological and biochemical analysis. After 4, 8, and 12 weeks of cultivation, extraction of 4 tendons was performed for biochemical analysis of ALP activity after cell lysis from the lysis overlap due to the fact that significant amounts of ALP are located within the cell membrane. Two additional tendons from each group were prepared for histology.

**Preparation of Tendons and Fixation in Cell Culture**

The tendons were explanted from the lower limbs of the bovines. Tendon preparation was performed under aseptic conditions. The tendons were explored, dissected, and washed in Earle’s solution (Biochrom AG, Berlin, Germany) for a period of 30 minutes. Fifty-mL Falcon tubes (Greiner Bio-One, Solingen, Germany) were used as reaction tubes. Tendons were cut into 2-cm pieces and washed again 3 times in modified Earle’s solution with antibiotics to minimize potential contamination (500 mL Earle’s solution + 250,000 IU penicillin/streptomycin + 2 mg amphotericin B). For long-term cultivation, additional tendon fixation was necessary. Therefore, the tendons were fixed with fibrin glue in Lumox cell culture dishes (Greiner Bio-One) and covered with modified BGJ medium (Sigma-Aldrich, Steinheim, Germany). The specimens were incubated with 5% CO₂ at 37°C.

**Cell Culture Nourishment**

For long-term cultivation, a modified standard BGJ medium was used. The medium was prepared according to manufacturer instructions with the additional use...
of fetal calf serum (FCS) and penicillin/streptomycin and the optional use of BMP-2 in groups 1 and 3.

The modified BGJ medium (Fitton-Jackson modification with L-glutamine) consists of 1.2 g/L of sodium bicarbonate, 5 mL/L (50,000 U/L) of penicillin/streptomycin, 5 mL/L (50 μg/mL) of gentamycin, 50 mL/L (10%) of FCS, 400 ng/mL of vitamin D3, and 400 ng/mL (groups 1 and 3) of BMP-2.

Every 48 hours, the cells were supplied with new medium. Once a week, a medium change with application of FCS-free solution was performed for 24 hours because FCS potentially contains fractions of ALP. Therefore, due to 24-hour cultivation with FCS-free medium, a more accurate analysis could be performed.

Biochemical Analysis

Once a week, the medium overlap was collected in 2-mL Eppendorf cups for analysis of ALP activity. Analysis was performed by an ALP assay (pNA reaction), which reacts as the following: pNA—phosphate+H₂O→free pNA+phosphate.

Alkaline Phosphatase Activity After Cell Lysis

Because significant amounts of ALP are located in the cell membrane, an additional ALP activity analysis out of cell lysis was performed once per week to make the dissociable proteins, such as ALP, measurable. The medium was extracted from the cell culture dishes. The dishes with the tendons were washed with phosphate-buffered saline (PBS), and then 2 mL of cold lysis PBS (6°C) were added for an additional 10 minutes. After centrifugation at 4°C with a rotation of 1600/min, the medium overlap was used to determine ALP activity according to the pNA reaction.

Histology

After 4, 8, and 12 weeks of cultivation, 2 tendons from each group were prepared for histology. The tendons were fixed in formalin, drained, and embedded in paraffin solution. Six-μm samples were cut using a microtome. Von Kossa stain was used to visualize potential micro calcification in the tendons. Hämalaun solution was used for counterstaining. Mineralized cartilage and bone appears dark brown or black with von Kossa staining.

Statistical Analysis

Statistical analysis was performed with SPSS version 17.0 software (SPSS, Inc, Chicago, Illinois). Mean±SD of ALP activity were calculated to show intergroup differences (groups 1-4). P values less than .05 using the Kruskal-Wallis test were considered significant.

RESULTS

Alkaline Phosphatase Activity Analysis

Alkaline phosphatase activity of the BMP-2–stimulated groups (groups 1 and 3) was higher over 10 weeks of the study period compared with the activity of the nonstimulated groups (groups 2 and 4). The highest increase in ALP activity was found in group 1, with BMP-2 stimulation and additional application of POBs. A significant increase (P<.001) was observed in the BMP-2–stimulated groups in week 2 of the experiment. Group 2 showed no relevant increases of ALP activity. In week 6, ALP activity began to decrease (P=.025). Adaptation of ALP activity among all groups was detected from week 8 onward, with no relevant differences regarding activity levels. Figure 1 shows an overview of all groups. Figure 2 shows the triggering effect of BMP-2 on ALP activity between groups 1 and 2 with additional application of POBs.

Alkaline Phosphatase Analysis Based on Cell Lysis

Analysis of ALP activity among groups with regard to cell lysis depending on BMP-2 stimulation showed equivalent results. The BMP-2–stimulated groups showed higher ALP activity at all measurement intervals. Alkaline phosphatase activity levels of group 1 decreased continuously and reached the lowest levels after 3 months. Alkaline phosphatase activity levels of group 2 were almost nonmeasurable, with peak levels reached after 2 months.

DISCUSSION

Tendon–bone integration continues to be a critical factor in ACL surgery and reconstructive rotator cuff surgery. Successful graft incorporation remains vitally important for clinical outcome. Firm attachment, graft remodeling, and graft incorporation at the tendon–bone interface are significant factors with regard to ACL-reconstruction. Recent research has focused on the integration processes of grafts in experimental animal models. The success of ACL reconstruction depends on initial graft fixation and subsequent biological integration of the graft to the bone.
on the current literature, BMPs and other growth factors are known to promote this process. Bone morphogenetic protein has been shown to be effective in promoting bone formation and tendon–bone healing in a bone tunnel. Rodeo et al. noted the enhancement of tendon healing within a bony tunnel after the application of recombinant human BMP-2. Furthermore, Martinek et al. supported these results because they showed that adenovirus–luciferase–transfected adenovirus–BMP-2 gene transfer significantly improved the integration of a double-bundle semitendinosus tendon graft after ACL reconstruction in a rabbit model. The goal of the current study was to evaluate the influence of BMP-2 on tendon–bone integration in an in vitro cell culture. The authors hypothesized that additional external application of BMP-2 may provide better and faster graft incorporation in vitro conditions, focusing on markers for graft incorporation, such as ALP. A successful in vitro cell culture model was established, which was confirmed by vital cells and evidence of cell proliferation on the bovine tendons after 12 weeks. Therefore, this model potentially enables long-term tendon cultivation and analysis of their characteristics based on an in vitro culture.

Alkaline Phosphatase Medium Overlap/Cell Lysis

Analysis of ALP activity in the medium overlap and cell lysis was higher in the BMP-2–stimulated subgroups than in the analogous controls over the period of cultivation. Positive triggering effects of BMP-2 and other BMPs on the activity of osteoblastic cell lines have been reported by other authors and were confirmed by the current study’s experimental setup. Wang et al. reported that combined recombinant human BMP-2 and fibroblast growth factor promoted significantly increased bone marrow stromal cell (BMSC) proliferation and differentiation of BMSCs compared with recombinant human BMP-2 or fibroblast growth factor alone. Their results show that a combination of recombinant human BMP-2 and fibroblast growth factor effectively induces early BMSC proliferation and differentiation in vitro. When combined, recombinant human BMP-2 and fibroblast growth factor synergistically promote new bone formation. Engstrand et al. reported similar results.

In the current study, analysis of differences in ALP activity among groups depending on BMP-2 stimulation showed overall higher ALP activity of the BMP-2–stimulated groups over the study period than of the nonstimulated groups. The highest increase in ALP activity was found in group 1, with BMP-2 stimulation and additional application of POBs. A significant increase (P<.001) was seen in the BMP-2–stimulated groups in week 2 of the experiment. After 6 weeks of cultivation, ALP activity began to decrease (P=.025). Adaptation of ALP activity among all groups was detected from week 8 onward, with no relevant differences regarding activity levels. Although no essential changes occurred in ALP activity in group 2, group 1 showed a substantial increase in ALP activity in week 2. According to the current literature, this initial peak function of BMP-2 is explained by the induction of osteoblast differentiation through Runx2-dependent unfolded pro-

Figure 1: Graph showing overall alkaline phosphatase (ALP) activity. The 2 groups that received bone morphogenetic protein-2 (BMP-2) every 48 hours had higher ALP levels than the other groups. Beginning in week 7, no relevant differences among groups 1 through 4 were detected. Medium and medium + osteoblasts served as controls. Abbreviation: t, time.

Figure 2: Graph comparing alkaline phosphatase (ALP) activity between groups 1 and 2 over 10 weeks. Group 1 (blue) received bone morphogenetic protein-2 (BMP-2) every 48 hours, and group 2 (red) did not. Both groups received additional primary bovine osteoblast application. Culture medium and osteoblasts with no BMP-2 and no tendon (fibroblasts) (green) served as a control. Significant correlation was set at a P value less than .001. Abbreviation: t, time.
tein response transducers, such as ATF6, a bZIP transcription factor within the BMP signaling cascade.\textsuperscript{13,31} Jang et al\textsuperscript{31} reported that BMP-2 activates unfolded protein response transducers, such as PERK and OASIS, in osteoblast cells in the early phase of tendon–bone integration.

The decrease in ALP activity in weeks 2 through 3 and 6 through 7 may be interpreted as follows: Based on studies by Canalis et al\textsuperscript{32} and Aspenberg et al,\textsuperscript{33} BMP inhibitors, such as Noggin and Chordin, may have been secreted by the osteoblasts to prevent an overwhelming ossification. These antagonists displace BMP from its receptor and block the processes that lead to transcription of target genes and, as a result, to differentiation of osteoblasts via Runx2-dependent signaling pathways.\textsuperscript{26,34} A potential possibility to avoid this antagonism is the use of BMP heterodimers. Zhu et al\textsuperscript{26} reported that BMP-2/7 heterodimers are more potent inducers of osteoblast differentiation as homodimers and induce significantly less expression of Noggin. The analysis of ALP activity after cell lysis depending on BMP-2 stimulation showed equivalent results. The BMP-2–stimulated groups showed higher ALP activities all measurement intervals. Peak levels with an activity of 12 U/L were detected in the BMP-2–stimulated groups. The decrease in the following period could be explained by the effects of the antagonists. Van Bezooijen et al\textsuperscript{34} reported the effect of sclerostin, which serves as a partial antagonist with regard to the osteoinductive capacities of BMPs. Compared with Noggin, sclerostin incorporates less specific affinity to specific BMPs (Noggin-specific inhibitor for BMP-2, -4, -7) but provides antagonism to all of the different BMP groups. Furthermore, its antagonizing capacities are time dependent.\textsuperscript{13,25,26,28}

Although the classical extracellular antagonists intervene in the early mineralization process, sclerostin is produced by the osteoblasts in the late stages of mineralization.\textsuperscript{26,34} Therefore, the continuous decrease of ALP activity could be explained by the osteoblast-induced antagonism of this mineralization process.

**Histology**

To detect the effects of BMP-2 on tendon–bone integration, von Kossa staining was used to visualize the process of tendon ossification for the duration of the 12-week period of cultivation. Histological analysis focused on the growth and formation of ECM and osteoid. After 4 weeks of cultivation in group 1, calcification-similar formations were detected, but analysis of the remaining groups showed disorganized cell accumulations of newly formed ECM with no evidence of calcification centers. The dominance of the first group continued after 8 and 12 weeks of cultivation. The histological results of group 1 match those of the ALP analysis (Figures 2, 4). Histological analysis showed progression of ECM formation and organization of the newly formed centers of calcification. This was not seen in the other groups.

The highest ALP activity in cell lysis and medium overlap were detected in the first month. High ALP values suggest high osteoblast activity levels and a high potential for mineralization. In the first month, the
precondition for a calcification could have been formed with clinical regard to tendon–bone integration. This early effect of BMP-2 was also reported by Yu et al.\textsuperscript{21} and other authors.\textsuperscript{10,12,14} Progression of newly formed ECM and cell nodules was detected from week 8 onward (Figure 3). At 12 weeks of cultivation, significant amounts of calcification centers were detected throughout the bovine tendons. Distribution showed different attachments to the surface of the tendon, representing different stages of already calcified parts of the tendon.

Furthermore, an increase in ECM formation occurred at the tendon boundary region in group 3 (Figure 3C). The newly formed boundary cell layer was remarkable compared with the ECM growth and formation in the other groups (Figures 3B, D). The effect became obvious in the third month. Calcium carbonate–like structures and ossification zones were detected only in the BMP–stimulated tendons. These findings concurred with those of Li et al.\textsuperscript{22} Group 3 also showed the second largest amount of ALP activity with regard to biochemical analysis (Figures 1, 2). According to the results of the ALP activity analysis and histological findings, BMP-2 may have an additional positive effect on fibroblasts as well as promoting overall tendon–bone integration.

Wang et al.\textsuperscript{24,25} reported that combined recombinant human BMP-2 and fibroblast growth factor promoted significantly increased BMSC proliferation and differentiation of BMSCs compared with recombinant human BMP-2 or fibroblast growth factor alone. Their results showed that, when combined, recombinant human BMP-2 and fibroblast growth factor synergistically promote new bone formation. These results support the theory that BMP-2 activates the fibroblasts and tendinocytes so that the tendon cells can play an active role in the process of tendon–bone integration.

In the current study, a successful in vitro cell culture model was established, which enabled long-term cultivation of tendons and analysis of their characteristics and interactions with growth factors based on an in vitro culture. However, the study uncovered some limitations. The study focused on the evaluation of BMP-2. Analysis and comparative analysis of other growth factors, especially in the BMP family, are warranted. Furthermore, only potential optimizing effects of BMP-2 on fibroblasts were detected. Based on the bovine tendon cultivation, further studies on the process of tendon–bone integration are warranted. Advanced clinical research is needed to prove the trends uncovered in this study.

**CONCLUSION**

The results of this study confirm the positive influence and triggering effect of BMP-2 on the mineralization process in a bovine in vitro model. Bone morphogenetic protein-2 seems to accelerate and optimize tendon–bone integration in the early process of graft incorporation. An additional positive effect of BMP-2 on bovine fibroblasts was detected; therefore, graft incorporation may be carried out by osteoblasts and fibroblasts.

**REFERENCES**


