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Brief communication

Inhibition of endogenous antagonists with an engineered BMP-2 variant increases BMP-2 efficacy in rat femoral defect healing

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ABSTRACT

Bone morphogenetic proteins (BMP) have been used successfully by orthopedic clinicians to augment bone healing. However, these osteoinductive proteins must be applied at high concentrations to induce bone formation. The limited therapeutic efficacy may be due to the local expression of BMP antagonists such as Noggin that neutralize exogenous and endogenous BMPs. If so, inhibiting BMP antagonists may provide an attractive option to augment BMP induced bone formation. The engineered BMP-2 variant L51P is deficient in BMP receptor type I binding, but maintains its affinity for BMP receptor type II and BMP antagonists including Noggin, Chordin and Gremlin. This modification makes L51P a BMP receptor-inactive inhibitor of BMP antagonists. We implanted β-tricalcium phosphate ceramics loaded with BMP-2 and/or L51P into a critical size defect model in the rat femur to investigate whether the inhibition of BMP antagonist with L51P enhances the therapeutic efficacy of exogenous BMP-2. Our study reveals that L51P reduces the demand of exogenous BMP-2 to induce bone healing markedly, without promoting bone formation directly when applied alone.

1. Introduction

Recombinant bone morphogenetic proteins such as BMP-2 and BMP-7 have been approved for clinical use to promote bone healing in tibia fractures, long bone non-unions and spinal fusions [1,2]. The growth factors, however, must be administered at concentrations that exceed those of naturally occurring BMPs present in 1000 kg of bone tissue [3]. This may be due to the *in vivo* regulation of the bioavailability of endogenous BMP by antagonistic proteins such as Noggin and members of the Dan and Chordin families [4,5]. Furthermore, BMPs themselves modulate the expression of various BMP regulatory proteins, including Noggin, Gremlin and Crossveinless-2 [6–8].

Expression of BMP antagonists increases during fracture healing and distraction osteogenesis [9–11]. Therapeutic requirements for administering high BMP concentrations to induce bone formation *in vivo* may result from significant neutralization of the growth factor by BMP inhibitory proteins. Thus, for clinical applications, the inhibition of BMP antagonists, rather than the direct application of BMPs, may present a preferred future strategy. L51P is an *in vitro* engineered BMP-2 variant with a leucine-to-proline substitution at codon 51. The modified protein is deficient in BMP type I receptor activation, but maintains its affinity for BMP type II receptors and inhibitory proteins such as Noggin, Gremlin and Chordin [12]. At equimolar concentrations, L51P blocks the inhibitory action of Noggin and restores BMP-2 dependent induction of alkaline phosphatase *in vitro* [12]. However, when added alone, L51P does not induce the differentiation of ATDC5 and C2C12-cells. Therefore, L51P represents a tool to enhance BMP-2-mediated bone formation *in vivo* through inhibition of endogenous BMP antagonists. Here, we determined whether the amount of BMP-2 required to induce bone formation could be reduced in the presence of L51P in a critical-size segmental defect in rat femur.

2. Methods

Ceramic β-tricalcium phosphate (β-TCP) cylinders (5 mm in diameter, 6 mm in length, 75% porosity) were provided by the Robert Mathys Foundation (RMS), Bettlach, Switzerland. Wistar rats (female, retired breeders, 350–450 g), purchased from Charles River (Sulzfeld, Germany), were housed in the Central Animal Facility of the Medical Faculty, University of Bern, Switzerland, in compliance with the Swiss Federal Government guidelines for care and use of experimental animals. The Bernese State Committee for the Control of Animal Experimentation approved our study

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(approval number 001/08 to WH). The BMP-2 and L51P proteins were expressed in *Escherichia coli*, refolded and purified as described previously [12–14].

For the measurement of the *in vitro* release of L51P and BMP-2 from the ceramic, 1 or 10 μg of BMP-2 or L51P was dissolved in 25 μl of deionized water and adsorbed to β-TCP carriers. After 24 h of drying, the loaded ceramics were incubated in 1.5 ml culture medium (alpha-minimum essential medium, 10% fetal bovine serum, 1% penicillin/streptomycin) for 19 days. The medium was changed after 24 h, and 4, 7, 10, 13 and 16 days. The amounts of BMP-2 and L51P released into the culture medium were quantified using an ELISA kit for the detection of BMP-2 (Human BMP-2 ELISA development kit, Peprotech, Rocky Hill, USA). This kit is suitable for the detection of L51P as well, as the structures of L51P and BMP-2 are identical except for the site of mutation.

For the *in vivo* experiments, BMP-2 and L51P were dissolved in 25 μl of deionized water and adsorbed to β-TCP carriers (Table 1). The loaded carriers were air-dried overnight and implanted into 6 mm critical-size segmental diaphyseal bone defects in rat femurs (seven groups, $n = 6$ per group). The defects were stabilized with the RatFix® (AO Foundation, Davos, Switzerland) osteosynthesis system. Migration of the implants, failure of the osteosyntheses and bone formation were monitored by X-ray imaging immediately after surgery and after 1, 4, 8 and 12 weeks. Implants were harvested together with the surrounding bone from animals sacrificed after 12 weeks. Bone formation was determined by quantitative histomorphometry on six serial 300 μm McNeal tetrachrome-stained ground sections (cross-sections) per implant. Data were analyzed statistically by one-way analysis of variance and the Sidak post-hoc test using SPSS® software for Mac (Version 16, SPSS Inc., Chicago, IL, USA).

3. Results

Evaluation of the *in vitro* protein release from β-TCP cylinders showed no difference in release kinetics between BMP-2 and L51P coatings. Furthermore, there was no difference in the relative protein release whether 1 or 10 μg of BMP-2/L51P was adsorbed to the ceramics. Adsorption of either 1 or 10 μg of both proteins resulted in a burst release of approximately 60% of the total protein amount within the first 24 h (Fig. 1).

Twelve weeks after implantation, no bone formation was observed when unloaded β-TCP cylinders (without proteins) or carriers adsorbed with 1 μg BMP-2 were implanted into the 6 mm rat femur defects (Figs. 2 and 3A). Ten micrograms of BMP-2 induced a significant increase in bone formation as compared to the unloaded carriers (bone volume/total volume (BV/TV): 10 μg BMP-2: 34.0 ± 1.9% vs. unloaded: 3.1 ± 1.4%, $p < 0.001$, Figs. 2 and 3B), resulting in the preservation of the original volume of the ceramic implant as seen in high resolution radiographs (Fig. 4B). Ten micrograms of L51P did not stimulate bone formation (Figs. 2 and 3C). When added to 1 μg BMP-2, L51P promoted bone formation in a dose-dependent manner. With 3 μg of L51P added to 1 μg BMP-2, a significant induction of bone formation over those carriers loaded with 1 μg BMP-2 alone was found (BV/TV: 3 μg L51P/1 μg BMP-2: 16.3 ± 6.1% vs. 1 μg BMP-2: 3.4 ± 0.7%, $p < 0.001$). Carriers loaded with 10 μg L51P and 1 μg BMP-2 induced bone formation

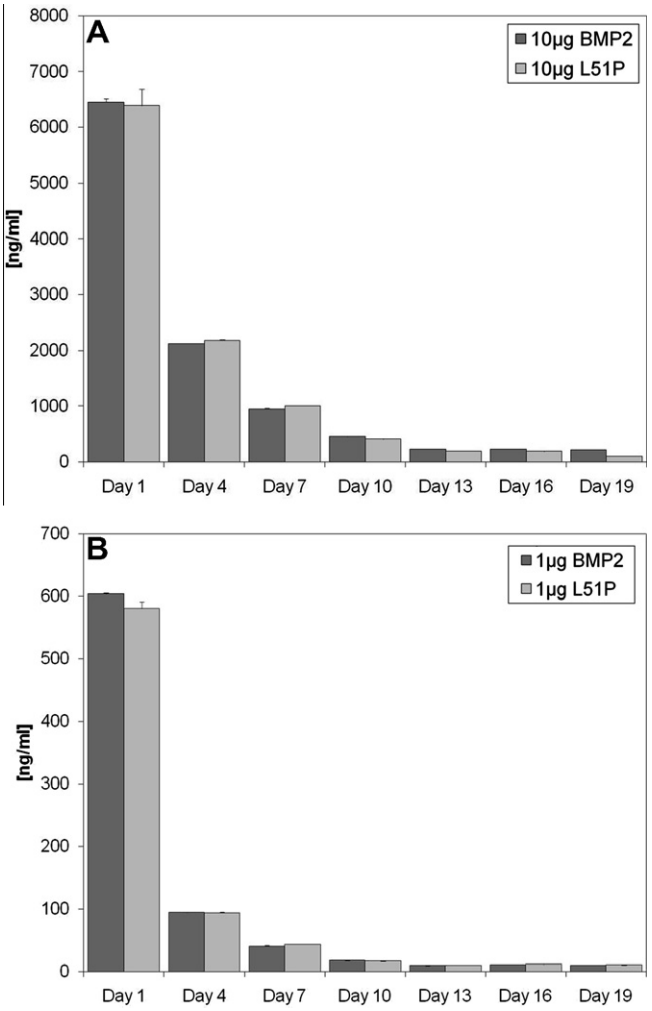

Fig. 1. Passive release kinetics of L51P and BMP-2 from β-TCP ceramics. BMP-2 or L51P (1 or 10 μg) was dissolved in 25 μl of deionized water and adsorbed to β-TCP cylinders. The ceramics were incubated in culture medium, and the BMP-2 and L51P release was measured after 24 h and after 4, 7, 10, 13, 16 and 19 days. (A) 10 μg L51P and BMP-2, (B) 1 μg L51P and BMP-2. Mean values are represented ± standard deviation ($n = 6$ for each group).

equivalently to carriers loaded with 10 μg BMP-2 (BV/TV: 10 μg L51P/1 μg BMP-2: 32.9 ± 6.7% vs. 10 μg BMP-2: 34.0 ± 1.9%). Bone formation was associated with degradation of the implanted carrier materials. Approximately 60% of the ceramic material degraded after 12 weeks in carriers loaded with 10 μg BMP-2. In carriers loaded with combinations of 1 μg BMP-2 and L51P, increasing the concentration of L51P induced β-TCP-degradation in a dose-dependent manner. Addition of 10 μg L51P to 1 μg BMP-2 resulted in similar material turnover as compared to materials loaded with 10 μg BMP-2 (material volume/total volume: 3 μg L51P/1 μg BMP-2 27.7 ± 6.9, 10 μg L51P/1 μg BMP-2: 17.6 ± 3.0% vs. 10 μg BMP-2: 13.5 ± 2.0%). Histologic transversal sections showed that the formation of new bone and the degradation of the carriers progressed from the center to the periphery of

Table 1
BMP-2 and L51P loading of β-TCP carriers.

Experimental groups						
I	II	III	IV	V	VI	VII
1 μg BMP-2	10 μg BMP-2	10 μg L51P	1 μg BMP-2 1 μg L51P	1 μg BMP-2 3 μg L51P	1 μg BMP-2 10 μg L51P	Unloaded