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

Hans-Jörg Sebalda, Frank M. Klenkea,⁎, Mark Siegristb, Christoph E. Albersb, Walter Sebd, Wilhelm Hofstetterb

aDepartment of Orthopedic Surgery, Inselspital, Bern University Hospital, CH-3010 Bern, Switzerland
bDepartment of Clinical Research, Group for Bone Biology and Orthopedic Research, University of Bern, CH-3010 Bern, Switzerland
cDepartment of Physiological Chemistry II, Biocenter, University of Würzburg, D-97074 Würzburg, Germany

⁎Corresponding author. Tel.: +41 31 632 2222; fax: +41 31 632 3600.
E-mail address: frank.klenke@dkf.unibe.ch (F.M. Klenke).

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.

© 2012 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
(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).

### 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 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 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

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μg BMP-2</td>
<td>10 μg BMP-2</td>
<td>10 μg L51P</td>
<td>1 μg BMP-2</td>
<td>1 μg L51P</td>
<td>1 μg BMP-2</td>
<td>1 μg BMP-2</td>
<td>1 μg BMP-2</td>
</tr>
</tbody>
</table>

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).
the materials. There was no bone formation found in the peripheral areas of the implants (Fig. 3E, F). After 12 weeks, high-resolution X-rays showed that the volume of the β-TCP ceramic implant upon adsorption with 1 μg BMP-2 and 10 μg L51P was preserved and that an interface between the host bone and the β-TCP ceramics was formed (Fig. 4F), similar to carriers loaded with 10 μg BMP-2 (Fig. 4B). Despite significant induction of bone formation, the defect volume was not maintained in carriers loaded with 1 μg BMP-2 and 3 μg L51P, and there was no formation of an interface between the implant and the host bone observed (Fig. 4E).

4. Discussion

Blocking the biological activities of BMP antagonists such as Noggin represents a promising strategy to stimulate bone formation [15]. Noggin levels in unstimulated osteoblasts are low but are strongly up-regulated by BMP-2 in vitro [6]. Expression of Noggin, Chordin, Gremlin and Dan was shown to be elevated during fracture healing in mice [9] and distraction osteogenesis in rats [10]. Noggin activity or expression has been successfully inhibited using cognate antibodies [8] and siRNA [16,17]. Furthermore, growth factor variants with reduced binding affinity to Noggin have been developed to enhance the osteogenic activity of BMPs [18,19]. Several groups were able to show that blocking Noggin [17] and Chordin [20] enhanced osteogenic differentiation by human mesenchymal stem cells and induced bone formation in vivo [17]. Our study did not aim at the identification of specific BMP antagonists limiting the activity of BMP-2. However, our results strongly suggest that endogenous BMP inhibitors limit exogenous BMP-2 activity in vivo, because L51P reconstituted the osteoinductive activity of otherwise ineffective BMP-2 concentrations.

L51P binds Noggin, Chordin, Crossveinless-2 and Gremlin, and possesses the same binding site for BMP-2 inhibitors as BMP-2 [12], suggesting that L51P binds and inhibits all known BMP-2 antagonists. In the present study, L51P promoted bone formation in critical size femur defects in rats by enhancing the activity of exogenously added BMP-2. L51P lacked osteoinductive activity when added alone, suggesting that the expression of endogenous
BMPs were not up-regulated beyond a critical threshold level necessary during the healing process of the applied critical-size defect model to induce the healing cascade. Relatively large amounts (3–10 μg) of L51P were necessary to rescue the osteogenic response to 1 μg BMP-2. The necessity to add high amounts of L51P is most likely due to high local concentrations of BMP-2 inhibitors. Exogenous BMP-2 results in a strong induction of Noggin and Chordin mRNA expression in osteoblasts [6,7]. We therefore suggest that the concentrations of BMP antagonists at the healing site surmount the concentration of BMP-2 by a molar ratio of greater than 3 to 10. Furthermore, the access of L51P to the local environment of the target cells may be limited and the availability of L51P restricted due to rapid degradation of the protein following release from the carrier.

5. Conclusion

This study shows that the efficacy of exogenous BMP-2 during in vivo bone defect healing – which by itself does not exert a biological effect when applied at 1 μg per implant – is improved by delivering an inhibitor of endogenous BMP antagonists. Furthermore, this study provides evidence that endogenous BMP antagonists play a major role in regulating BMP actions during bone repair. It is preferable to introduce an inhibitor of BMP antagonists into a bone defect site instead of active BMP-2, since exogenous BMP-2 is not controlled by cellular mechanisms and may induce side effects such as heterotopic ossification if the protein cannot be contained at the repair side. In contrast, improving the bioavailability of BMP-2 and increasing its efficacy by blocking BMP antagonists with L51P allows BMP-2 levels and activities to be regulated by the cellular environment. The protein delivery-based approach with β-tricalcium phosphate ceramics reported here will allow for the use of smaller doses of exogenous BMP-2 to augment bone regeneration and may lead to an easy-to-handle “off-the-shelf” clinical product not requiring intraoperative implant preparation. Further long-term studies with biomechanical testing are warranted to investigate whether the combination of small BMP-2 doses and L51P does induce complete substitution of β-TCP ceramics by authentic bone and whether this results in mechanically competent defect bridging.

Acknowledgements

We thank the Robert Mathys Foundation (RMS), Bettlach, Switzerland for providing custom-made β-tricalcium phosphate cylinders.

References


