L51P — A BMP2 variant with osteoinductive activity via inhibition of Noggin

Christoph E. Albers, Wilhelm Hofstetter, Hans-Jörg Sebalb, Walter Sebald, Klaus A. Siebenrock, Frank M. Klenke

A Group for Bone Biology and Orthopedic Research, Department of Clinical Research, University of Bern, CH-3010 Bern, Switzerland
b Department of Orthopedic Surgery, Inselspital, Bern University Hospital, CH-3010 Bern, Switzerland
c Department of Physiological Chemistry II, Biocenter, University of Würzburg, D-97074 Würzburg, Germany

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ABSTRACT

Bone morphogenetic proteins (BMP) have to be applied at high concentrations to stimulate bone healing. The limited therapeutic efficacy may be due to the local presence of BMP antagonists such as Noggin. Thus, inhibiting BMP antagonists is an attractive therapeutic option. We hypothesized that the engineered BMP2 variant L51P stimulates osteoinduction by antagonizing Noggin-mediated inhibition of BMP2. Primary murine osteoblasts (OB) were treated with L51P, BMP2, and Noggin. OB proliferation and differentiation were quantified with XTT and alkaline phosphatase (ALP) assays. BMP receptor dependent intracellular signaling in OB was evaluated with Smad and p38 MAPK phosphorylation assays. BMP2, Noggin, BMP receptor Ia/Ib/II, osteocalcin, and ALP mRNA expressions were analyzed with real-time PCR. L51P stimulated OB differentiation by blocking Noggin mediated inhibition of BMP2. L51P did not isolate BMP dependent intracellular signaling via the Smad pathway. Treatment of OB cultures with BMP2 but not with L51P resulted in an increased expression of ALP, BMP2, and Noggin mRNA. By inhibiting the BMP antagonist Noggin, L51P enhances BMP2 activity and stimulates osteoinduction without exhibiting direct osteoinductive function. Indirect osteoinduction with L51P seems to be advantageous to osteoinduction with BMP2 as BMP2 stimulates the expression of Noggin thereby self-limiting its own osteoinductive activity. Treatment with L51P is the first protein-based approach available to augment BMP2 induced bone regeneration through inhibition of BMP antagonists. The described strategy may help to decrease the amounts of exogenous BMPs currently required to stimulate bone healing.

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Introduction

Recombinant bone morphogenetic protein 2 (BMP2) has been used in orthopedics to augment bone healing in tibia fractures, long bone non-unions and spinal fusion. However, BMP2 has to be used at levels that exceed those of naturally occurring BMP2 present in 1000 kg of bone tissue to induce sufficient bone formation [1–5].

BMP signaling is regulated by antagonistic proteins such as Noggin, Chordin, Gremlin, and Dan [6,7]. These extracellular antagonists bind to BMPs, block the proteins’ receptor binding epitopes to BMPR-IA/B and BMPR-II, and inhibit the formation of ligand–receptor complexes [8–11]. The expression of the BMP antagonists is increased during fracture healing and distraction osteogenesis [12–14]. The need to administer high concentrations of exogenous BMPs to induce bone formation may be the result of the inhibitory activity of BMP antagonists [6,15].

The key role of antagonists in the action of BMPs suggests that inactivating these antagonists may enhance BMP signaling during bone regeneration. Keller et al. [16] previously reported the synthesis of an engineered BMP2 variant called L51P. L51P is deficient in type I receptor binding, whereas its overall structure and its binding to type II receptors and modulator proteins, including Noggin, Gremlin, and Chordin, are unchanged. These modifications make L51P a BMP receptor-inactive inhibitor of endogenous BMP antagonists [16]. The concept of antagonizing endogenous BMP inhibitors with L51P is a promising option to augment BMP induced bone regeneration. Here, we investigated whether L51P enhances BMP2 mediated osteoinduction in primary osteoblasts via inhibition of Noggin and whether L51P preserves direct osteoinductive activity in the absence of BMP2.

Materials and methods

Cell cultures

Primary osteoblasts were isolated from calvariae of 1–2-day-old ddY mice according to Takahashi et al. [17–19] by sequential collagenase digestion [20] and subsequently stored in liquid nitrogen at 1 × 10⁶ cells/ml. Thereafter, cells were thawed and expanded for 4 days in α-MEM (26 mM bicarbonate, Gibco, Basel, Switzerland) supplemented with 10% FBS (heat inactivated, Gibco, Basel, Switzerland) and 1% Penicillin G/Streptomycin (Pen/Strep, Gibco, Basel, Switzerland).
Cell cultures were treated with L51P (synthesized at the Department of Physiological Chemistry, University of Würzburg, Germany), BMP2 (synthesized at the Department of Physiological Chemistry, University of Würzburg, Germany), BMP2 + Noggin, (PeproTech Inc., Rocky Hill, NJ 08553, USA, Cat No. 120-10C), L51P + Noggin, and BMP2 + Noggin + L51P at increasing concentrations for cell proliferation and differentiation assays (0 nM, 2 nM, 4 nM, 8 nM, 16 nM, 32 nM, 64 nM, 128 nM, and 256 nM) and at equimolar concentrations (32 nM) for the assessment of intracellular signaling and gene expression studies. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. Culture media were changed after 72 h.

Osteoblast proliferation and differentiation

For the assessment of cell proliferation and differentiation, 2 × 10³ cells were seeded in 96-well plates and were grown for 6 days in α-MEM (26 mM bicarbonate) supplemented with 10% FBS (heat inactivated) and 1% Pen/Strep and treated with BMP2, L51P, and/or Noggin as described in Cell cultures, the treatments starting on day 0. XTT assays were used to assess osteoblast proliferation (Cell proliferation kit II, Roche Diagnostics, Rotkreuz, Switzerland). 50 μl XTT labeling mixture was added into each cell culture. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ for 180 min. The absorbance was measured at 490 nm (Infinite M200, Tecan Austria GmbH, Grödig, Austria). Osteoblast differentiation was assessed by determining alkaline phosphatase (ALP) activity. Directly after performing XTT assays, the cultures were washed with PBS and lysed with 25 μl 0.1% Triton X-100 (Sigma Aldrich, T8787) followed by three cycles of freezing and thawing. Afterwards, 25 μl of 4-nitrophenyl phosphate (Sigma Aldrich, N3254) (1 mg/ml) in 1 M diethanolamine (pH 9.8) was added. The reaction was stopped after 30 min with 0.1 M EDTA (Merck 1.08418); 150 mM NaCl (Merck 1.06404); 1 mM NaF (Merck 1.06449); 1 mM activated Na3VO4; 25 μg/ml protease inhibitor cocktail (Sigma Aldrich, p8340); and 0.5% NP-40 (Sigma Aldrich, 9016-45-9); pH 8.0). Total protein content was determined using the Bio-Rad Protein Assay (Cat. No.: 500-0006). Samples were then subjected to 10% SDS-PAGE (Criterion Tris–HCl Gel, 10%, 26-well) and transferred to polyvinylidene difluoride transfer membranes (Amersham, RPN 20 F). Non-specific binding sites were blocked by immersing the membranes in 10% skim milk in TBS for 120 min at room temperature, then the membrane was washed several times with TBS, followed by incubation with the primary antibody at 4 °C for 12 h. Anti-Smad 1/5/8 (Santa Cruz Biotechnology, Heidelberg, Germany, Cat. No. cs-6031-R), anti-phospho-Smad 1/5/8 (Cell Signaling Technology, Danvers, MA, USA, Cat. No. 9511), anti-p38 MAP Kinase (Cell Signaling Technology, Danvers, MA, USA, Cat. No. 9212), and anti-phospho-p38

![Fig. 1. Primary murine osteoblasts were treated with 0–128 nM (A) BMP2, (B) L51P, (C) BMP2 + Noggin, and (D) BMP2 + Noggin + L51P over 6 days. After 6 days of culture, osteoblast differentiation and proliferation were measured with ALP and XTT assays, respectively. Optical densities in ALP assays were normalized to the cell number obtained from XTT assays. Mean±SD. *p<0.001 versus 0 nM.](image-url)
MAP Kinase (Cell Signaling Technology, Danvers, MA, USA, Cat. No. 9211) were used as primary antibodies. Horseradish peroxidase (HRP)-linked anti-rabbit IgG served as secondary antibody (GE Healthcare, Switzerland, Cat. No. NA934). Lumigen® TMA-6 (Lumigen Inc., USA, Cat. No. TMA-100) was the substrate for the chemiluminescent detection of HRP conjugates. Chemiluminescence was detected with a VersaDoc Imaging System (Model 3000, 7681-49-4, Bio-Rad). Smad 1/5/8 phosphorylation was analyzed semiquantitatively by measuring the gray value intensity of the blots using Adobe Photoshop CS5 for Mac OS X (Adobe Systems Inc., San Jose, USA) according to the following equation: 255 (gray value for white at 8 bit resolution) minus measured gray value.

Real-time PCR

To determine the levels of transcripts encoding Noggin, BMP2, BMP-receptor (BMPR)-Ia/b, BMPR-II, Bglap and ALP in murine osteoblasts, cells were seeded into 12-well plates (2×10^4 cells/well), grown for 6 days, and treated with BMP2, L51P, and/or Noggin as described in Cell cultures, starting on day 0. Total RNA was isolated using the RNeasy Mini Kit from Qiagen (Cat. no. 74106), following the recommendations of the manufacturer. Total RNA was reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, M1705), random primers (Promega, C1181), and nucleotide mix (Roche, 11814362001), and RNase inhibitors (Roche: 03335402001). Total RNA was quantified spectrophotometrically (Thermo Scientific NanoDrop2000 Spectrometer, Software v1.4.1). Real-time PCR was performed in an ABI 7500 System (Applied Biosystems, Software SDS v1.4.0.25), using Assays-on-Demand (Applied Biosystems) for Noggin (Mm99999915_g1), BMP2 (Mm01340178_m1), BMP-receptor (BMPR)-Ia (Mm00475831_m1), BMPR-II (Mm00432134_m1), Bglap (Mm03413826_mH), and ALP (Mm01340178_m1), BMP-receptor (BMPR)-Ia/b, BMPR-II, Bglap and ALP in murine osteoblasts. Mean±SD (*p < 0.05) were found. The corresponding levels of unsphosphorylated Smad 1/5/8 were similar in all experimental conditions. Investigation of p38 MAPK did not show phosphorylation of p38 in any experimental condition; viz: treatment of murine osteoblasts either with BMP2, L51P, or BMP2+Noggin did not show phosphorylation of p38 in any experimental condition.

Statistical analysis

For all analyses three independent experiments were performed; ALP and XTT assays were conducted with n = 6 per experiment and experimental group; Western blots and real-time PCR were conducted with n = 3 per experiment and experimental group. The data demonstrated were chosen from a single representative experiment. All data are presented as means ± standard deviation from individual experiments. Data were analyzed statistically by one-way ANOVA and Sidak post-hoc test using SPSS® software for Mac (Version 16, SPSS Inc., Chicago, IL, USA).

Results

Effects of L51P on osteoblast differentiation

Primary murine osteoblasts were treated with increasing concentrations of (i) L51P, (ii) BMP2, (iii) BMP2 + Noggin, (iv) L51P + Noggin (data not shown, identical to L51P only), (v) BMP2 + L51P (data not shown, identical to BMP2 only), and (vi) BMP2 + Noggin + L51P. Osteoblast differentiation was stimulated by BMP2 in a dose dependent manner (Fig. 1A). No stimulatory effects on osteoblasts were observed when the cells were treated with L51P alone (Fig. 1B). BMP2 mediated osteoblast differentiation was antagonized by Noggin in a dose dependent manner. Complete inhibition of BMP2 induced osteoblast differentiation was achieved with equimolar (32 nM) and higher concentrations of Noggin (Fig. 1C). L51P added to osteoblasts treated with 32 nM BMP2 and 32 nM Noggin blocked the inhibitory action of Noggin in a dose dependent manner. At concentrations of 32 nM and higher, L51P neutralized the activity of Noggin completely (Fig. 1D).

Activation of intracellular signaling by BMP2 and L51P

Western blots were carried out to assess the effects of BMP2 and L51P on BMPR-II receptor dependent intracellular signaling via Smad1/5/8 and p38 MAPK. BMP2 induced the Smad signaling pathway in murine osteoblasts as demonstrated by the phosphorylation of Smad1/5/8 (Fig. 2A). There was no phosphorylation of Smad1/5/8 found when osteoblasts were treated with L51P alone. Noggin inhibited BMP2 dependent Smad1/5/8 phosphorylation at equimolar concentrations (32 nM). Equimolar concentrations of L51P restored Smad signaling in osteoblasts when BMP2 induced Smad1/5/8 phosphorylation was inhibited with Noggin. Semiquantitative analysis showed that the levels of phosphorylated Smad1/5/8 did not differ among negative controls, cells treated with 32 nM L51P, and cells treated with 32 nM BMP2 plus 32 nM Noggin (Fig. 2B). In cells treated with 32 nM BMP2 or 32 nM BMP2, L51P, and Noggin, increased levels of phosphorylated Smad1/5/8 as compared to negative controls (175.4 ± 14.8 vs. 116.1 ± 12.2, p < 0.05) and as compared to cells treated with 32 nM BMP2 plus 32 nM Noggin (168.1 ± 19.4 vs. 121.1 ± 10.8, p < 0.05) were found. The corresponding levels of unsphosphorylated Smad 1/5/8 were similar in all experimental conditions. Investigation of p38 MAPK did not show phosphorylation of p38 in any experimental condition; viz: treatment of murine osteoblasts either with BMP2, L51P, or BMP2+Noggin did not show phosphorylation of p38 in any experimental condition.
Noggin or one of the applied combinations of the factors did not result in an induction of p38 phosphorylation (data not shown).

**Gene expression in osteoblasts upon stimulation with BMP2 and L51P**

The effects of BMP2 and L51P on BMP2, Noggin, BMPRIa/II, Bglap, and ALP mRNA levels in murine osteoblasts were investigated. BMP2 induced the expression of BMP2, Noggin, ALP and Bgalp mRNA (Fig. 3), while no BMP2 dependent increase of levels of transcripts encoding BMPRI-la, BMPRI-lb and BMPRI-II were detected in comparison to negative controls (untreated cells) (Fig. 4). The induction of Noggin mRNA expression after simulation with 32 nM BMP2 was 24.5 fold higher than the induction of BMP2 mRNA expression (Noggin $2^{\Delta\Delta CT}$: 403.3, BMP2 $2^{\Delta\Delta CT}$: 16.6, p < 0.001). BMP2 mediated increase of BMP2, Noggin, Bglap and ALP transcript levels was completely antagonized after the addition of Noggin at equimolar concentrations (32 nM). Treatment with L51P alone did not have stimulatory or inhibitory effects on the expression of the evaluated genes as compared to the negative controls (untreated cells). When osteoblasts were treated with BMP2, Noggin and L51P at equimolar concentrations (32 nM), L51P blocked the Noggin mediated inhibition of BMP2 induced gene expression. The values of BMP2, Noggin, ALP and Bgalp mRNA were not significantly different when osteoblasts were treated with 32 nM BMP2 or 32 nM BMP2, 32 nM Noggin, and 32 nM L51P (BMP2 mRNA: 16.5 ± 1.5 vs. 17.3 ± 2.8, p = 0.764; Noggin mRNA: 403.3 ± 21.7 vs. 479.4 ± 32.6, p = 0.129; ALP mRNA 662.1 ± 130.3 vs. 559.2 ± 58.3, p = 0.453; Bglap mRNA: 115.6 ± 7.8 vs. 88.4 ± 12.1, p = 0.137).

**Discussion**

The inhibition of BMP antagonists represents a promising option to enhance the efficacy of BMP in bone healing. L51P inhibits Noggin, which regulates the signaling of BMP2, BMP4, and BMP7, and might be a candidate to augment BMP induced bone healing [8,16]. Furthermore, the inhibition of BMP antagonists regulating several BMPs may result in a more efficient and physiological osteoinductive response than does the delivery of a single exogenous osteoinductive factor.

Suppression of Noggin to enhance BMP signaling has been attempted previously using small interfering RNA (siRNA). Takayama et al. showed that Noggin silencing enhanced BMP2 induced osteoblast differentiation in C2C12 cells and BMP2 induced ectopic bone formation in mice [21]. Suppression of Noggin expression in MC3T3-E1 cells and calvarial osteoblasts by means of siRNA resulted in enhanced osteogenic differentiation without further addition of BMPs [22]. Calvarial osteoblasts transfected with Noggin suppressing siRNA accelerated the healing of murine calvarial defects when compared to non-transfected osteoblasts [22]. These results indicate that Noggin suppression by siRNA enhances the osteoinductive activity of endogenous and exogenous BMPs. However, the application of siRNA in vivo is complicated.
Osteoblasts have to be harvested from the host, transfected with siRNA constructs \textit{ex vivo} by adenovirus mediated gene transfer, and then re-implanted into the host \cite{22}. Alternatively, Noggin directed siRNA may be injected into the site of required bone regeneration and cellular uptake of siRNA may be achieved with electroporation \cite{21}.

To the best of our knowledge this is the first study reporting a protein-based approach to enhance BMP2-mediated osteoinduction via inhibition of Noggin. We show that the BMP2 variant L51P itself has no osteoinductive activity as demonstrated by the absent induction of ALP activity as well as the deficient upregulation of ALP and Bglap mRNA in murine osteoblasts. The osteoinductive function of L51P depends on the presence of BMP2 and Noggin. At equimolar concentrations, L51P completely neutralized the Noggin-mediated inhibition of ALP activity in BMP2 stimulated osteoblasts. Furthermore, L51P reconstituted the expression of ALP and Bglap in osteoblasts inhibited with Noggin to similar levels as those observed when osteoblasts were stimulated with BMP2 alone. Although late stage osteoblast differentiation markers such as osteocalcin and osteopontin \cite{23–26} were not analyzed at the protein level, these results indicate that L51P does not have direct effects on osteoblast differentiation but mediates its osteoinductive function indirectly via the inhibition of Noggin. As previously shown by Keller et al. \cite{16} L51P has a higher binding affinity to Noggin than wild-type BMP2. Therefore, L51P functions as a competitor for Noggin reducing its bioavailability to bind and inhibit BMP2.

Transduction of BMP signaling is mediated through hetero-dimeric receptor complexes composed of type I and type II transmembrane serine/threonine kinase receptors \cite{27}. BMP-receptor binding has been described as occurring through preformed or through induced type I/II receptor complexes. Binding of BMP2 to BMPR-I and subsequent formation of hetero-dimeric receptor complexes with BMPR-II induces intracellular signaling via the p38 pathway \cite{28}. Alternatively, BMP2 may bind to preformed BMPR-I/-II receptor complexes activating the Smad pathway through phosphorylation of Smad1/5/8 \cite{28}.

We therefore investigated the induction of MAPK and Smad phosphorylation by L51P. In our cell culture system we did not find an up-regulation of phosphorylated p38 upon treatment with BMP2, L51P, and Noggin in any experimental condition. Thus, we cannot draw a conclusion on the effects of L51P on the p38 pathway. However, as L51P is deficient in type 1 receptor binding it seems unlikely that the protein mediates BMP signaling via the p38 pathway \cite{16}. L51P itself did not induce the phosphorylation of Smad1/5/8 in osteoblasts showing that the modified protein does not mediate BMP2 signaling via the Smad pathway. However, Noggin mediated inhibition of BMP2-receptor dependent activation of the Smad pathway was blocked by simultaneous treatment with L51P. While total Smad1/5/8 protein levels were not observed to change, an increase in phosphorylated Smad1/5/8 was found when Noggin was antagonized by L51P indicating that the inhibition of Noggin with L51P augments intracellular BMP2 dependent signaling via the Smad pathway.

Several studies have investigated gene expression patterns in osteoblasts upon stimulation with BMP2 including Noggin, BMP2, ALP, Bglap and BMP receptors \cite{29–34}. In accordance with previous studies we found an increase in the expression of Noggin and BMP2 mRNA in response to stimulation with BMP2 \cite{35}. The up-regulation of Noggin was 24.5 fold higher than the up-regulation of BMP2 suggesting a shift in the ratio of Noggin and BMP2 towards inhibitory Noggin activity. In contrast, L51P inhibits the BMP2 antagonist Noggin without upregulating endogenous Noggin expression. Neither BMP2 nor L51P had an impact on the expression of BMPR-Ia, BMPR-Ib and BMPR-II in murine osteoblasts showing that the abrogated binding capacity of L51P to BMPR-I did not change the receptor status of the cells.

**Conclusions**

L51P does not exhibit direct osteoinductive activity \textit{in vitro} but stimulates osteoinduction \textit{via} competitive inhibition of Noggin, thereby reducing the bioavailability of Noggin to inhibit BMP2. Enhancing BMP2 activity via inhibition of extracellular BMP antagonists seems to be advantageous to direct osteoinduction with BMP2. As Noggin
regulates several BMPs its inhibition may result in a more efficient and physiological osteoinductive response than does the delivery of a single exogenous BMP. Furthermore, exogenous BMP2 stimulates the expression of Noggin self-limiting its osteoinductive activity. Increasing the biological activity of endogenous and exogenous BMPs with L51P is a promising tool to augment bone regeneration. L51P may decrease the high amounts of exogenous BMP2 currently required to stimulate bone healing. Further studies are warranted to investigate the potential of L51P to enhance bone healing in vivo.

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