Journal of Bone and Mineral Metabolism (2019) 37:199–205
https://doi.org/10.1007/s00774-018-0925-0

REVIEW ARTICLE

The BMP-2 mutant L51P: a BMP receptor IA binding-deficient inhibitor of noggin

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Received: 1 February 2018 / Accepted: 1 April 2018 / Published online: 17 April 2018

Abstract

The antagonist-specific regulation in tissue engineering constitutes important attempts to achieve an improved and rapid bone regeneration by controlling the natural biological response of the natural body growth factors. L51P is molecularly engineered bone morphogentic protein-2 (BMP-2) variant with a substitution of the 51st leucine with a proline residue. L51P is deficient in BMP receptor binding, but maintains its structure and affinity for inhibitory proteins such as noggin, chordin, and gremlin. These modifications convert the BMP-2 variant L51P into a receptor-inactive inhibitor of BMP antagonists. This current approach may prevent the uncontrolled bone overgrowth using high concentration of BMPs and thus regulates the possible growth factor's high-dose side effects. Exploring of L51P biological functions is required to broad our understanding of BMP mutant biological functions and their potential clinical applications. The progress of L51P researches would hopefully lead to the development of multiple applications for using the L51P in bone and fracture healing disorders.

Keywords BMP-2 · BMP antagonist · L51P · BMP negative feedback

Abbreviation

BMP	Bone morphogenetic protein
BRI	BMP receptor type I
BRII	BMP receptor type II
CCN	Cysteine-rich 61, connective tissue growth factor, nephroblastoma-overexpressed

Introduction

Bone morphogenetic proteins (BMPs) are cytokines that play a crucial role in osteogenesis and bone formation during development and regeneration after tissue damage. Ectopic BMP administration is used to enhance local bone regeneration in humans [1, 2]. BMP signaling is initially activated when BMPs bind to the heterotetrameric complexes of type I and type II serine/threonine kinase receptors found on the surface of almost all of the normal cells. Recruitment of the intracellular signaling molecules is initiated through the activation of type I receptors, and the SMAD phosphorylation pathway results in transcription of early target genes. Compelling evidence suggests that BMP antagonists precisely regulate BMPs [3]. Noggin, follistatin, gremlin 1, and chordin are BMP antagonists that modulate their function through the direct association with BMPs barring them from binding to their receptors [4]. However, other antagonists such as sclerostin may inhibit the SMAD cascade by binding to the downstream signaling proteins [5]. In large mammals and humans, high doses of BMPs are required to induce sufficient bone healing [6]. Indeed, milligram doses of BMPs are needed to induce sufficient bone repair, whereas only nanogram doses of BMPs are normally present in the human body. To improve such a current BMP treatment, there is an urgent need to find novel strategies to enhance the efficiency

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of BMPs by combating the antagonists [7, 8]. L51P had previously emerged as an in vitro engineered BMP-2 variant with a leucine to proline substitution at codon 51. By this mutation, L51P lost one of the central hydrogen bonds due to the amino acid substitution, leading to the loss of the high binding affinity to BMP receptor type IA (BRIA). On the other hand, L51P retains the wild-type affinity to the BMP modulator proteins, such as noggin and gremlin [9, 10]. This article provides a brief review of L51P protein function and biological actions, and discusses how this BMP-2 mutant could be used as novel therapeutic approaches in modulating BMP antagonists. A schematic model is presented to summarize the mode of action of L51P (Fig. 1) [11]. BMP signals can be blocked by extracellular antagonists that bind to BMP ligands, which prevent their association with the BMP receptor (Fig. 1a). L51P specifically binds to BMP antagonists, allowing BMP signaling to proceed to osteoinduction (Fig. 1b). The timing of the targeting of BMP-2 antagonists may be crucial. The delayed addition of L51P may encourage the receptor activation by BMPs and significantly strengthening the signaling, probably because this mutant would diminish the levels of free BMP antagonists in the extracellular microenvironment, as explained in detail later (Fig. 1c). The strategy of inactivation of BMP antagonists by L51P may provide a better therapeutic option to enhance bone regeneration, rather than the administration of high concentrations of BMPs that hold their own side effects [12].

Proline substitution in the main chain of the amide group of BMP-2 reveals a binding hot spot

The intermolecular hydrogen bonds in a single BMP-2/BRIA were consistently indicated using the potential hydrogen bond calculator; HBPLUS [13]. Many possible main and side-chain hydrogen bonds seem to exist at the interface between BMP-2 ligands and BRIA ligand binding domain. Among ten indicated intermolecular hydrogen bonds of BMP-2, BMP-2 shares five main-chain atoms for hydrogen bonding, whereas the receptor shares four. Accordingly, the proline substitution of the main-chain atom Leu 51 of BMP-2 increased the dissociation constant (Kd) between L51P and BRIA up to 8000 times higher [9]. The obvious high dissociation constant certainly represents the markedly lower affinity of L51P to the ligand binding domain of BMP receptor type I (BRI). In contrast, the other main-chain hydrogen bonds have only minor effects on the binding affinities between BMP-2 and BRI. These observations implicated that the main-chain hydrogen bond disturbed by the proline substitution is a major binding determinant to BRI [9]. Importantly, the affinity of this proline variant to BMP receptor type II (BRII) is comparable to the wild type. The proline substitution of BMP-2 forming L51P mutant distorted the space used earlier by hydrogen bond between BMP-2 Leu51 amide and the side-chain carbonyl of the extracellular domain of BRI (BRIEC) Gln86 that presents

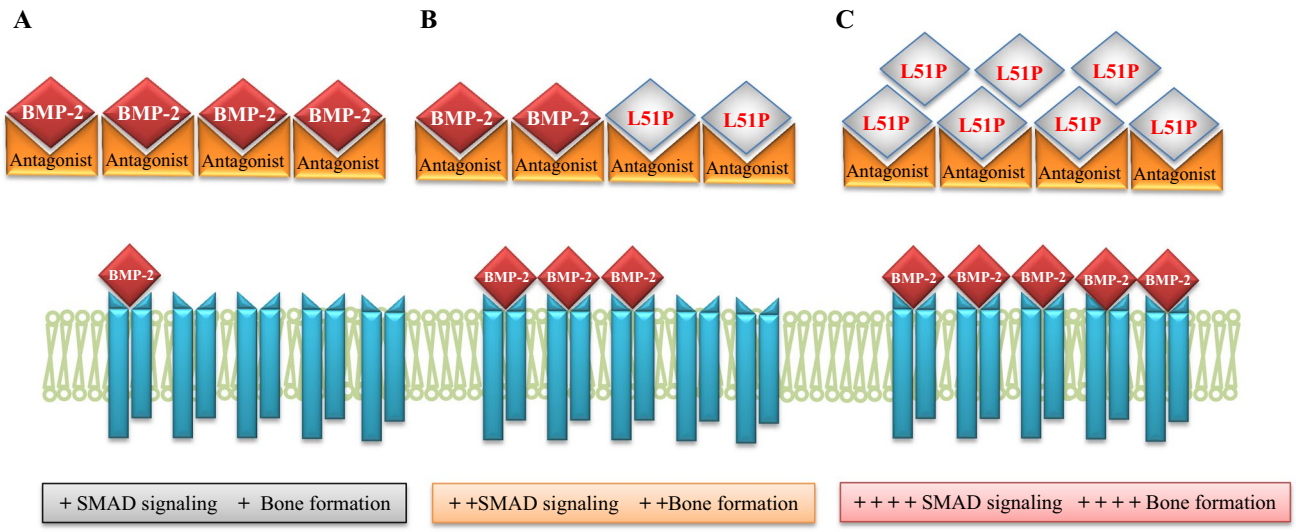

Fig. 1 A schematic diagram showing BMP signaling and the possible action of L51P. BMP initially binds to type I and type II receptors, which subsequently phosphorylate a member of the SMAD family that translocates to the nucleus and up regulates BMP early-response genes, leading to increased bone formation. **a** The effect of BMP antagonists on BMP signaling subsequently downregulates bone formation. **b** Trapping such antagonists by L51P may result in promotion of the interaction between BMP ligands and their receptors, resulting in enhanced osteogenesis. **c** Delayed targeting of BMP antagonists may effectively counteract the normal negative feedback action exerted on BMP-2 ligands, contributing to a significant increase in the osteogenic potential of BMP-2

steric hindrance. Therefore, the side chain of L51P pushed back further to accommodate Pro51 in the interface between BMP-2 and BRI [9]. This pushback causes the backbone carbonyl of Pro51 to move toward the side chain of BRIEC Gln86 (Fig. 2). Consequently, the huge loss of binding energy by the mutation L51P cannot be avoided. Furthermore, another study of BMP-2 variants showed the presence of double or triple mutants of BMP-2, in which the knuckle epitope mutations were introduced in the background of the L51P mutation. These mutants showed a dramatically reduced affinity to BRII as well as BRI [14].

Biological activity of L51P

L51P has no osteoinductive activity, which was mainly evaluated by alkaline phosphatase (ALP) assays with primary murine osteoblasts, MC3T3-E1, ATDC5, and promyoblast [15–17]. L51P could neutralize the inhibitory effects of noggin on BMP-2-induced ALP activity in these works. Under the BMP-2 unstimulated conditions, a moderate dose of L51P could not affect the levels of endogenous BMP-2 and/or BMP antagonists. However, it should be noted that in cells such as nucleus pulposus cells (NPC) and annulus fibrosus cells (AFC) that are characterized by a marked endogenous expression of BMP antagonists, L51P alone could restore the osteogenic mineralization lost, by blocking the activity of the BMP antagonists secreted by the former cells [18].

Previously, Keller et al. [9] observed that ALP activity in ATDC5 cells was not enhanced when 250 nM of L51P was applied to the cells. However, in the study of Khattab et al. [17], a significant increase in the ALP activity was observed after 5 μg stimulation with L51P under BMP-2 unstimulated conditions. This finding can be explained by extensive abolishment of the intrinsic BMP-2 antagonists, as the endogenous BMP-2 pathway was believed to be activated to some extent. Although this may be true, it is also possible that the atom substitution of the BMP-2 amino acid mutant L51P, which led to a mutant with reduced affinity to its receptor, had a change in the protein's three-dimensional shape, which could ignite the BMP signaling even under the complete absence of BMP-2 [19].

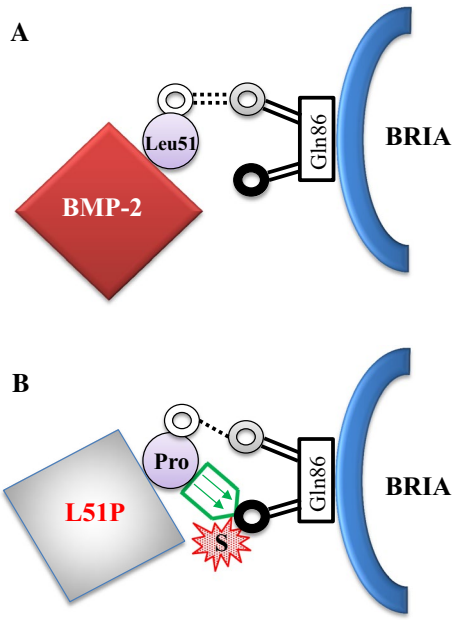

Fig. 2 A schematic diagram showing the interaction among amino acid residues involved in the BMP-2 and L51P variant, which explains the steric hindrance induced by the mutation. **a** The major hydrogen bond between wild-type BMP-2 and BRIA Gln86 is shown by dashed lines. **b** Green arrow indicating the movement of carbonyl of Pro51 toward the side chain of BRIEC Gln86, thus replacement of Leu51 by proline forming L51P is likely to cause the steric hindrance (shown by letter S) between proline and the Gln86 of BRIA, which drastically reduce the energy of the hydrogen bond supporting the interaction, as explained in the text

L51P effects on the canonical and non-canonical BMP signaling pathways

According to a previous report [16], BMP-2 and BMP-2/L51P simultaneous combination groups showed equal activation of p-SMAD-1/5/8 even after 48 h of stimulation. On the other hand, treatment with L51P alone did not activate SMAD signaling. It is widely recognized that, in addition to the SMAD-mediated canonical BMP signaling pathway, BMP-2 can also activate non-canonical mitogen-activated protein kinase (MAPK) pathways, where BMP-2 activates the p38, extracellular signal-regulated kinase (ERK) 1/2, and *c-jun* N-terminal kinase (JNK) 1/2 signaling pathways [20–24]. Previously, the experimental observations by Albers et al. have demonstrated that 10 min stimulation with BMP-2 and L51P cannot initiate p38 phosphorylation [15]. The authors also obtained consistent results up to 30 min after the stimulation either with BMP-2 (100 ng/ml) or L51P (10, 100 ng/ml). In addition, our group also found that BMP-2 and L51P did not influence the downstream signaling initiated by JNK and ERK1/2 in MC3T3-E1. These results suggest that L51P does not initiate BMP-2 signaling via these MAPKs. Yet, other trials showed that p38 phosphorylation occurs following BMP-2 stimulation in mouse and human osteoblastic cells [25–27]. Together with the data that were observed from our laboratory and from others, we are not able to draw a scientific scheme explaining how BMP-2 as well as L51P could influence p38 pathway. It is also possible that the activation of the non-canonical pathway could be initiated in later time points from that we examined.

L51P in vivo

In the in vivo evaluation by Sebald et al. [16], BMP-2/L51P combination could induce bone regeneration within 12 weeks in critical-sized rat femur defect with ceramic β-tricalcium phosphate carriers. Loading of 10 µg of L51P and 1 µg of BMP-2 to the carrier showed the same results compared to the carrier loaded with 10 µg of BMP-2. In this experiment, the protein release kinetic assay reveled that approximately 60% of the total protein was released from the carrier within 24 h. Nevertheless, the ceramic β-tricalciumphosphate carriers cannot control the growth factor release [16]. In the rat critical-sized in vivo defect model by our group [17], we used a carrier that has been found to be efficient for the long-term delivery of growth factors [28]. The adsorption and retention of L51P and BMP-2 were confirmed by protein-loaded biodegradable hydrogels that were subcutaneously implanted in 10-week-old mice for 2 weeks. 5 µg of BMP-2 and 1 µg of L51P combination over 4 weeks induced a significantly higher degree of bone formation than 5 µg of BMP-2. These findings suggest that using a carrier aiding, a prolonged protein retention would enhance the efficiency of osteoinduction by L51P with BMP-2.

Delayed addition of L51P to BMP-2

The increased expression of the BMP antagonists during healing may require higher exogenous BMP concentration to promote bone healing [5, 29]. There have been no reports on the direct cellular actions of delayed apposition of noggin and/or other BMP-2 antagonists on the BMP-2 osteoinductive effects. Since the mRNA expression of BMP antagonists subsequent to BMP-2 stimulation suggests local feedback mechanisms controlling BMP-2 function, these antagonists probably act by binding to BMP-2 secreted by osteoblasts. BMP-2 stimulation caused a time- and dose-dependent induction of Nog mRNA for 2 h, which sustained for 24 h in primary murine osteoblasts [15]. In addition, BMP-2 also increased noggin mRNA levels in the MC3T3-E1 osteoblastic cell line [17]. The Kd values of BMP-2 and L51P to noggin and chordin are quite similar and are not affected by the proline substitution [9]. It is reasonable to think that stimulation with L51P in a delayed method after BMP-2 stimulation, at the same time of the expected BMP-2 antagonist induction, may be more practical for L51P to act against these antagonists. Our group showed that [17], when cells were pre-treated with BMP-2 for 3 days, there was an increase in the mRNA expression levels of BMP-2 antagonists such as Nog, Grem 1, and Chord. It was hypothesized that the delayed addition of L51P would significantly enhance the ability of BMP-2 to induce bone formation [17]. The delayed addition of L51P after BMP-2 stimulation, dramatically enhanced SMAD signaling, and promoted their osteogenic differentiation compared to cells treated with BMP-2 and L51P simultaneously [17]. Therefore, the co-operation between BMP-2 and L51P with time interval may provide a novel therapeutic approach for the clinical enhancement of bone formation (Fig. 1c). Likewise, in the intervertebral disc (IVD) cells that abundantly secrete the major BMP antagonists, such as noggin, gremlin 1, and chordin, the concentration of the secreted BMP antagonists was capable of preventing the osteogenic differentiation potential of mesenchymal stem cells (MSCs) [18] and the osteoblastic differentiation [30]. Targeting of the BMP antagonists by addition of L51P to the co-cultures of MSC and IVD cells that plentifully secrete the BMP antagonists appeared to be significant. Tekari and his group successfully showed that L51P could recover the osteogenic differentiation of MSCs in the presence of IVD cells [18]. Similarly, CCN3 is one of the antagonists of BMP-2 [31–34]. In the bone regeneration model with *Ccn3*-null mice (Ccn3 KO), the expression levels of Nog, Chord, and Grem1 were significantly increased after 5 and 10 days of drill hole injury in the mice femurs than those in wild-type mice [32]. Thus, in the Ccn3 KO mice model, the acceleration of the bone regeneration after L51P administration alone after 5 or 10 days of femoral osteotomy may provide a strong evidence of direct targeting of L51P to BMP antagonists. The control of the timing of L51P application may provide a new research avenue leading to L51P/BMP co-operative regeneration therapeutics.

Other noggin-resistant BMPs

Noggin was found to play an important role in the negative feedback regulation of BMPs. Therefore, BMPs that are insensitive to noggin may provide a unique molecular tissue engineering with rhBMPs (recombinant BMPs) with noggin antagonism resistance [35]. Song et al. [36] investigated the noggin negative feedback regulation of BMP-6 and BMP-7, and the results showed a different phosphorylation of SMAD-1 in BMP-6-treated C2C12 cells and MC3T3 preosteoblasts from that with BMP-7. In addition, they observed that BMP-4 was the most sensitive to noggin, followed by BMP-2 and then BMP-7, whereas BMP-6 showed the highest resistance to noggin in the experiments with ROS 17/2.8 cells. These results indicated that the differential responses of different BMPs to noggin resulted from the different intrinsic characteristics of various BMPs. In opposite to L51P, one of the artificial BMP-6 chimera 80-1

showed a prominent resistance to noggin inhibition. Here, it is worthy to note the findings reported by Seeman et al. [37], which describe noggin-sensitive BMPs. It is described that the co-expression of noggin did not suppress the chondrogenic effects of BMP-9 and BMP-10. The BMP-9 mutant K348L rendered them completely to nog-sensitive without affecting the chondrogenic potential in the absence of noggin [37]. Therefore, manipulating the affinities of BMPs to noggin may also provide a novel source for optimal BMP therapeutics for clinical applications.

L51P potential research prospects

L51P and CCN2

The CCN family of proteins is a complex family of multifunctional cysteine-rich secretory proteins that interact with a number of growth factors, including BMPs [38]. [39–42]. Studies revealed that CCN2 interacts with BMP-2 and BMP-4 through its von Willebrand type C and C-terminal domains [39, 40]. In this context, there is evidence that CCN2 interacts with BMP-4 preventing the ligands from binding to its associated receptor BRIA, thus inhibiting BMP signaling [39]. The combination of BMP-2 and CCN2 suppressed phosphorylation of SMAD (p-SMAD)-1/5/8 induced by BMP-2 in cultured chondrocytes, leading to the suppression of proliferation and stimulation of differentiation of the chondrocytes [40]. Osteoblasts that over-express CCN2 have also decreased the protein levels of p-SMAD-1/5/8, suggesting that CTGF/CCN2 regulates the BMP signaling pathway [43, 44]. Collectively, these data suggest that CCN2 may act as a negative regulator for BMP signaling in a way similar to those of BMP antagonists. However, the current situation regarding the CCN2 effect on osteoblast differentiation is controversial [40, 43–45]. Our group previously reported that the combination of BMP-2 and CCN2 had an additive promoting effect on chondrocyte differentiation [40]. Because it is well known that CCN2 shows multiple functions, which are supposed to be mediated by different domains, therefore, CCN2 can have promoting and inhibiting effects on the differentiation of osteoblasts via different mechanisms through different domains, depending upon the microenvironment [40, 43–46]. In addition, it is well known that the process of wound healing is regulated by a complex network of cytokines. These cytokines include transforming growth factor-β (TGF-β) and CCN proteins [47]. In fibroblasts, CCN2 is induced by TGF-β and thus is considered a downstream mediator of certain TGF-β effects leading to fibrotic lesion formation in diverse organs and tissues [48, 49], and the degree of over-expression defines the severity of disease [50]. L51P would interact with modulator proteins (CCN2 proteins) and that could alter the modulatory effect of such modulator protein to a different manner compared to the modulator protein alone. BMPs have a chondro-protective effect in different animal models on articular cartilage [51, 52]. Thus, so far as the CCN-interface possibly is retained in L51P, and together with the data presenting the high expression and the pathological role of CCN genes in rheumatoid arthritis (RA) samples, [53] these data suggest a possible value of L51P in exploring pathophysiology of RA.

L51P and osteoporosis

Sclerostin (SOST) was originally identified by positional cloning of the disease-causing gene in sclerosteosis. It is widely known that the inhibition of sclerostin leads to increased bone density, definitely making sclerostin and its signaling pathway as interesting targets for the development of bone anabolic agents against osteoporosis [54, 55]. SOST was found to have significant binding affinity to BMP-6, BMP-7, BMP-2, and BMP-4 with dissociation constants of 5.7, 87.6, 100, and 224 nM, respectively [56]. However, it is believed that sclerostin is biologically a canonical Wnt antagonist and its effects on bone mass are primarily attributed to its antagonistic effect on canonical Wnt signaling. In addition, there is a big variance in the binding affinities of BMPs to noggin (1 pM) and sclerostin (100 nM) [57]. Many studies showed that sclerostin expression is precisely confined to osteoblasts in both endochondral and intramembranous bones, where BMPs are playing critical roles [57–59]. Therefore, L51P as well as BMP-2 may affect the function of sclerostin via direct molecular interaction. In this scenario, L51P may provide a novel clue for the treatment of clinical cases with reduced bone density, such as osteoporosis.

Acknowledgements The author would like to thank all his colleagues in the Department of Oral Rehabilitation and Regenerative Medicine, the Department of Biochemistry and Molecular Dentistry, and the Department of Advanced Research Center for Oral and Craniofacial Sciences, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan, for their generous hosting and all kind assistances during the time taken to accomplish this review. The author also would like to thank Dr. Kristin Sainani A. Professor at Stanford University for her technical assistance.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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