Alendronate Inhibits PTH (1–34)-induced Bone Morphogenetic Protein Expression in MC3T3-E1 Preosteoblastic Cells

Paul S. Issack, MD, PhD · Margaret H. Lauerma, BS · David L. Helfet, MD · Stephen B. Doty, PhD · Joseph M. Lane, MD

Published online: 31 March 2007
© Hospital for Special Surgery 2007

Abstract The bisphosphonate class of antiresorptive drugs and active forms of parathyroid hormone (PTH (1–34)) have been used clinically to enhance bone mass and density in patients with osteoporosis. Abundant evidence suggests that the mechanism by which PTH (1–34) increases bone density is stimulation of osteoblast differentiation. Although bisphosphonates have been classically thought to increase bone density by inhibiting osteoclasts, there is increasing evidence to suggest that bisphosphonates have direct stimulatory effects on osteoblast differentiation. Interestingly, in patients with osteoporosis, combination therapy with bisphosphonates and PTH (1–34) is not synergistic in increasing bone density; bisphosphonates appear to blunt the effect of PTH (1–34). To begin to understand the mechanism governing the effects of these agents on osteoblasts and a possible explanation for their apparent antagonism, we examined the expression of several bone morphogenetic proteins (BMPs) in MC3T3-E1 preosteoblastic cells either untreated, or treated with alendronate, parathyroid hormone, or a combination of the two agents. We find by reverse transcriptase-polymerase chain reaction (RT-PCR) that while alendronate fails to induce the expression of any of the BMPs tested, several BMPs are induced by PTH (1–34). The induction of the PTH (1–34)-inducible BMPs is blocked with simultaneous alendronate treatment. These data suggest that alendronate interferes with PTH (1–34)-induced BMP gene transcription and provides a possible basis for the antagonism observed between the two agents in increasing bone density.

Key words osteoblasts · bisphosphonates · parathyroid hormone · BMPs

Introduction

Bone remodeling involves both resorption and bone formation—coupled processes that occur throughout life. When this highly regulated linkage is disrupted, the structural and material properties of bone diminish. Several human diseases, including postmenopausal osteoporosis, Paget's disease, and fibrous dysplasia result from this uncoupling of bone formation and bone resorption. Low bone mineral density with increased risk of fragility and pathologic fractures results when the rate of resorption exceeds the rate of formation [1, 2].

Bisphosphonate drugs such as alendronate have been used to restore the balance between bone resorption and bone formation [1, 2]. There is abundant evidence suggesting that bisphosphonates act to decrease bone resorption by inhibiting the function of osteoclasts [1]. More recently, there has been increasing evidence to suggest that bisphosphonates may act to stimulate osteoblast differentiation [3–7]. Whereas the mechanism governing bisphosphonate-mediated osteogenesis is poorly understood, the evidence that alendronate can increase the expression of osteoblast-specific marker genes such as osteocalcin and type I collagen suggests that this regulation may be, in part, at the transcriptional level [7].

Parathyroid hormone (PTH) is an anabolic agent that enhances osteoblastic bone formation. Teriparatide, the active N-terminal fragment of PTH (residues 1–34), has been approved by the US Food and Drug Administration for the treatment of postmenopausal women with osteoporosis who are at high risk for fracture [2]. Because bisphosphonates have been shown to inhibit osteoclasts, a mechanism distinct from the anabolic effect of PTH (1–34) on osteoblasts, it has been suggested that their effects in osteoporosis may be additive. However, two clinical trials of the active form of PTH (1–34) and alendronate in men and postmenopausal women demonstrated that alendronate appeared to impair the ability of the active form of PTH (1–34) to increase bone mineral density [8–10]. The mechanism governing the antagonistic effects of combination therapy is unclear.

P. S. Issack, MD, PhD · M. H. Lauerma, BS · D. L. Helfet, MD · S. B. Doty, PhD · J. M. Lane, MD (✉)
Hospital for Special Surgery,
535 East 70th Street, New York, NY 10021, USA
e-mail: lanej@hss.edu

Bone morphogenetic proteins, including BMP-2, BMP-4, and BMP-7, play critical roles in osteoblast differentiation and bone formation and are thus good candidates for mediating the osteogenic signals of bisphosphonates and PTH (1–34) [11]. We therefore examined the expression of several bone morphogenetic proteins in osteoblasts either untreated or treated with alendronate, PTH (1–34), or a combination of the two agents. We are using the MC3T3-E1 preosteoblastic cell line, which has been widely used as a cell culture model for osteoblast differentiation. In response to several osteogenic signals such as the bone morphogenetic proteins or ascorbic acid, these cells express osteoblast-specific genes such as osteocalcin, core-binding factor alpha subunit 1 (Cbfa1), bone sialoprotein II, and type I collagen and undergo morphologic changes including mineralization [12–17]. We find by RT-PCR that BMP-1, which is not a member of the BMP/TGF- β family, and BMP-4 are constitutively expressed in untreated MC3T3-E1 cells, and treatment with alendronate does not alter this expression pattern. Treatment of MC3T3-E1 cells with PTH (1–34) induces expression of several of the BMPs; this induction is blocked by simultaneous alendronate treatment. Understanding the interaction of differentiation signals initiated by alendronate and PTH (1–34) will help provide a molecular basis for the design of better combination therapies to more effectively treat disorders of bone insufficiency.

Materials and methods

Materials and cell culture

PTH (1–34) (Forteo) containing the biologically active N-terminal 34 residues of parathyroid hormone was obtained from Eli Lilly (Indianapolis, IN, USA). Alendronate (Fosamax) was obtained from Merck and Co. (Whitehouse Station, NJ, USA). MC3T3-E1 preosteoblastic cells were cultured at 37°C, 10% CO₂, in 60-mm dishes, in α -MEM (Invitrogen) supplemented with 10% fetal bovine serum (Sigma) and 50 μ g/ml penicillin and streptomycin. Cells were treated with either alendronate (10^{-8}), PTH (1–34) (10^{-7}), or both, 24 h after plating (at 80% confluence). There was no additional media change upon treatment. Cells were harvested for RNA isolation 24 h after treatment.

RNA isolation and RT-PCR

Total RNA was isolated using Trizol Reagent (Invitrogen). Reverse transcriptase-PCR (RT-PCR) was performed using a reverse transcription kit (ReactionReady first strand cDNA synthesis kit; Super Array, Frederick, MD, USA) and a PCR kit consisting of primers for mouse BMP genes (mouse BMP gene family multigene-12 RT-PCR profiling kit, PM-040B; Super Array, Fredrick, MD, USA). RT-PCR was performed according to the manufacturer's instructions. PCR was performed using a thermal cycler (Applied Biosystems, Foster City, CA, USA). Ten microliters of PCR product was analyzed on a 2.0% agarose gel.

Results

Alendronate inhibits mRNA expression of several BMPs in PTH (1–34) treated MC3T3-E1 osteoblasts

We examined expression of several BMPs by RT-PCR in MC3T3-E1 cells treated with either alendronate (10^{-8}), PTH (1–34) (10^{-7}), or both (Fig. 1A–D). Concentrations were chosen based on published dose-response studies [7, 18, 19]. We note that BMP-1 and BMP-4 are constitutively expressed in MC3T3E1 cells (Fig. 1A). Alendronate does not induce expression of any of the BMPs over untreated MC3T3E1 cells (Fig. 1B). PTH (1–34) induces several BMPs including BMP-2, 3, 5, 6, 7, 8A, 8B, 10, and 3B (Fig. 1C). Interestingly, combination treatment with PTH (1–34) and alendronate blocks the mRNA expression of all nine PTH (1–34)-inducible BMPs (Fig. 1D).

Discussion

The ability of bisphosphonates to inhibit osteoclasts coupled with the stimulatory effects of PTH (1–34) on osteoblasts suggests that their effects in increasing bone mineral density may be additive. However, recent clinical trials have demonstrated that alendronate appeared to impair the ability of PTH (1–34) to increase bone mineral density [8–10]. We observe that alendronate blocks the ability of PTH (1–34) to induce the mRNA expression of several BMPs in the preosteoblastic MC3T3-E1 cell line. This suggests a mechanism for the observed clinical antagonism between alendronate and PTH (1–34) in increasing bone density.

The BMPs encode a highly conserved class of signaling factors, which can induce ectopic bone formation *in vivo*. They are expressed during limb development, endochondral ossification, and early fracture healing. In several animal studies, BMPs have demonstrated the ability to enhance spinal fusion, repair critical-size defects, and accelerate fracture union [20, 21]. Interestingly, we find that BMP-1, distinct from the TGF- β -like BMPs, is

Fig. 1. RT-PCR for expression of BMP family members (BMP-1, 2, 3, 4, 5, 6, 7, 8A, 8B, 10, 3B) and GAPDH (lanes 1–12, respectively) in MC3T3-E1 cells either untreated (A) or treated with alendronate [10^{-8}], (B) PTH (1–34) [10^{-7}] (C), or alendronate [10^{-8}] and PTH (1–34) [10^{-7}] (D). PCR products were resolved by 2.0% agarose gel electrophoresis

constitutively expressed. BMP-1 is a tolloid-like proteinase, which plays critical roles in the synthesis of extracellular matrix proteins including several types of collagens and proteoglycans [22]. BMP-1 activates latent TGF- β by cleaving latent TGF- β -binding protein [23] and can modulate BMP-2 and BMP-4 signaling [24]. Constitutive expression of BMP-1 in this cell type may reflect the preosteoblastic genotype of these cells and their potential to differentiate down the osteoblastic pathway.

Two BMPs have been widely studied for their ability to induce bone regeneration in the clinical situation: recombinant human BMP-2 (rhBMP-2) and recombinant human BMP-7 (OP-1 [osteogenic protein-1]). These factors induce bone formation and healing of bone when surgically introduced [20, 21, 25]. In clinical trials, rhBMP-2 and OP-1 have been shown to accelerate the healing of open tibial fractures and tibial nonunions, respectively [26, 27]. The induction of BMP-2 and BMP-7 by PTH (1–34) is consistent with the bone-forming capacity of these agents and may partly explain the inductive effect of PTH on osteoblast differentiation. The block of this induction by alendronate can, in part, account for the antagonism observed between bisphosphonates and PTH (1–34) in increasing bone formation.

While increased BMP expression in PTH-treated MC3T3-E1 cells is suggestive of increased osteogenic potential, expression of other bone markers must be examined. There is evidence that PTH stimulates osteogenesis and increases expression of the bone-specific gene osteocalcin by increasing activity of the transcription factor osteoblast-specific factor 1 (osf1) [19]. If PTH regulates this process by induction of BMPs, future studies would be required to examine the ability of BMP antagonists to block the ability of PTH to increase osteocalcin gene transcription; this would confirm the necessity of BMP to transduce the PTH-mediated osteogenic signal.

Conclusion

The mechanism by which alendronate suppresses PTH (1–34)-induced BMP expression is unclear. The lack of expression of mRNAs for PTH (1–34)-inducible BMPs in MC3T3 E1 cells when treated with both PTH (1–34) and alendronate suggests that alendronate represses expression of these BMPs at the transcriptional level. Future studies aimed at identifying regions in the BMP-2 and BMP-7 promoters repressed by simultaneous alendronate and PTH (1–34) treatment and comparing these regions to PTH (1–34)-inducible elements in these promoters may provide insight into the early PTH (1–34)-mediated signal pathways interrupted by alendronate. Understanding the interactions of these signaling pathways is a first step toward designing superior combination therapies for treating low bone density and preventing pathologic fractures.

Acknowledgment This study was funded in part by grants from Eli Lilly (Indianapolis, IN, USA).

References

1. Lin JT, Lane JM (2003) Bisphosphonates. *J Am Acad Orthop Surg* 11:1–4
2. Madore GR, Sherman PJ, Lane JM (2004) Parathyroid hormone. *J Am Acad Orthop Surg* 12:67–71
3. Bukowski JF, Dascher CC, Das H (2005) Alternative bisphosphonate targets and mechanisms of action. *Biochem Biophys Res Commun* 328:746–750
4. Giuliani N, Pedrazzoni M, Negri G et al (1998) Bisphosphonates stimulate formation of osteoblast precursors and mineralized nodules in murine and human bone marrow cultures in vitro and promote early osteoblastogenesis in young and aged mice in vivo. *Bone* 22:455–461
5. Abe Y, Kawakami A, Nakashima T et al (2000) Etidronate inhibits human osteoblast apoptosis by inhibition of pro-apoptotic factor(s) produced by activated T cells. *J Lab Clin Med* 136:344–354
6. Plotkin LI, Weinstein RS, Parfitt AM et al (1999) Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. *J Clin Invest* 104:1363–1374
7. von Knoch F, Jaquiere C, Kowalsky M et al (2005) Effects of bisphosphonates on proliferation and osteoblast differentiation of human bone marrow stromal cells. *Biomaterials* 26:6941–6949
8. Black DM, Greenspan SL, Ensrud KE et al (2003) The effects of parathyroid hormone and alendronate alone or in combination in postmenopausal osteoporosis. *N Engl J Med* 349:1207–1215
9. Finkelstein JS, Hayes A, Hunzelman JL et al (2003) The effects of parathyroid hormone, alendronate, or both in men with osteoporosis. *N Engl J Med* 349:1216–1226
10. Lecart MP, Bruyere O, Reginster JY (2004) Combination/sequential therapy in osteoporosis. *Curr Osteoporos Rep* 2:123–130
11. Reddi AH (2001) Bone morphogenetic proteins: from basic science to clinical applications. *J Bone Jt Surg Am* 83-A(Suppl 1): S1–S6
12. Gopalakrishnan R, Jiang D, Reith E et al (2002) Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells. *J Bone Miner Res* 17:101–110
13. Noda M, Rodan GA (1986) Type-beta transforming growth factor inhibits proliferation and expression of alkaline phosphatase in murine osteoblast-like cells. *Biochem Biophys Res Commun* 140:56–65
14. Noda M, Vogel R (1989) Fibroblast growth factor enhances type beta 1 transforming growth factor gene expression in osteoblast-like cells. *J Cell Biol* 109:2529–2535
15. Tsuji K, Ito Y, Noda M (1998) Expression of the PEBP2alphaA/AML3/CBFA1 gene is regulated by BMP4/7 heterodimer and its overexpression suppresses type I collagen and osteocalcin gene expression in osteoblastic and nonosteoblastic mesenchymal cells. *Bone* 22:87–92
16. Xiao G, Cui Y, Ducy P et al (1997) Ascorbic acid-dependent activation of the osteocalcin promoter in MC3T3-E1 preosteoblasts: requirement for collagen matrix synthesis and the presence of an intact OSE2 sequence. *Mol Endocrinol* 11:1103–1113
17. Xiao G, Jiang D, Gopalakrishnan R et al (2002) Fibroblast growth factor 2 induction of the osteocalcin gene requires MAPK activity and phosphorylation of the osteoblast transcription factor, Cbfa1/Runx2. *J Biol Chem* 277:36181–36187
18. Im GI, Qureshi SA, Kenney J et al (2004) Osteoblast proliferation and maturation by bisphosphonates. *Biomaterials* 25:4105–4115
19. Jiang D, Franceschi RT, Boules H et al (2004) Parathyroid hormone induction of the osteocalcin gene. Requirement for an osteoblast-specific element 1 sequence in the promoter and involvement of multiple-signaling pathways. *J Biol Chem* 279: 5329–5337
20. Issack PS, DiCesare PE (2003) Recent advances toward the clinical application of bone morphogenetic proteins in bone and cartilage repair. *Am J Orthop* 32:429–436

21. Wozney JM, Rosen V (1998) Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair. *Clin Orthop Relat Res* 346:26–37
22. Ge G, Greenspan DS (2006) Developmental roles of the BMP1/TLD metalloproteinases. *Birth Defects Res C Embryo Today* 78:47–68
23. Ge G, Greenspan DS (2006) BMP1 controls TGF β 1 activation via cleavage of latent TGF β -binding protein. *J Cell Biol* 175:111–120
24. Jasuja R, Ge G, Voss NG et al (2007) Bone morphogenetic protein 1 prodomain specifically binds and regulates signaling by bone morphogenetic proteins 2 and 4. *J Biol Chem* 282:9053–9062
25. Einhorn TA, Majeska RJ, Mohaideen A et al (2003) A single percutaneous injection of recombinant human bone morphogenetic protein-2 accelerates fracture repair. *J Bone Jt Surg Am* 85-A(Suppl):1425–1435
26. Friedlaender GE, Perry CR, Cole JD et al (2001) Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions. *J Bone Jt Surg Am* 83-A(Suppl):S151–S158
27. Govender S, Csimma C, Genant HK et al (2002) Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients. *J Bone Jt Surg Am* 84-A:2123–2134