Sclerostin Inhibition of Wnt-3a-induced C3H10T1/2 Cell Differentiation Is Indirect and Mediated by Bone Morphogenetic Proteins*

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High bone mass diseases are caused both by activating mutations in the Wnt pathway and by loss of SOST, a bone morphogenetic protein (BMP) antagonist, leading to the activation of BMP signaling. Given the phenotypic similarity between mutations that activate these signaling pathways, it seems likely that BMPs and Wnts operate in parallel or represent components of the same pathway, modulating osteoblast differentiation. In this study, we show that in C3H10T1/2 cells, Wnt-3a and BMP-6 proteins were inducers of osteoblast differentiation, as measured by alkaline phosphatase (ALP) induction. Surprisingly, sclerostin, noggin, and human BMP receptor 1A (BMPR1A)-FC fusion proteins blocked Wnt-3a-induced ALP as well as BMP-6-induced ALP activity. Dkk-1, a Wnt inhibitor, blocked Wnt-induced ALP activity but not BMP-induced ALP activity. Early Wnt-3a signaling as measured by β-catenin accumulation was not affected by the BMP antagonists but was blocked by Dkk-1. Wnt-3a induced the appearance of BMP-4 mRNA 12 h prior to that of ALP in C3H10T1/2 cells. We propose that sclerostin and other BMP antagonists do not block Wnt signaling directly. Sclerostin blocks Wnt-induced ALP activity by blocking the activity of BMP proteins produced by Wnt treatment. The expression of BMP proteins in this autocrine loop is essential for Wnt-3a-induced osteoblast differentiation.

Sclerostin, the protein product of the sost gene associated with the high bone mass sclerosteosis phenotype, was predicted to be a secreted glycoprotein with homology to the DAN family of bone morphogenetic protein (BMP) antagonists and more distantly to the BMP antagonist noggin (1, 4). We have shown that sclerostin behaved as a BMP antagonist and blocked BMP-induced osteoblastic activity such as alkaline phosphatase (ALP) activity in human and rodent bone cell models (3). In a broader context, BMPs and BMP antagonists have described skeletal roles specifically in chondrocyte differentiation, joint formation, and osteogenesis (5–7). Recently, Fischer et al. (8) and Bain et al. (9) reported that BMP-2 mediated osteoblast differentiation was modulated by a member of the Wnt protein family (8, 9). A novel role for Wnts was implicated in osteogenesis and BMP signaling.

Wnt proteins are cysteine-rich, secreted glycoproteins that have been implicated in embryonic development and cellular differentiation, in particular limb patterning and chondrogenesis (10, 11). Wnt proteins activate downstream signaling pathways in target cells through interactions with Frizzled and low density lipoprotein receptors (LRP) co-receptors. The identification of the high bone mass gene, an activating mutation in LRPs, revealed the role of the Wnt pathway in bone formation (2, 12).

Given the interaction between the BMP and Wnt proteins, we were interested in investigating whether BMP antagonists, in particular sclerostin, could modify Wnt activity. Our findings show that the sclerostin antagonist of Wnt-3a-induced activity was not due to a direct interaction between the proteins or between sclerostin and the Wnt signaling pathway. We conclude that sclerostin inhibition of Wnt activity is mediated by BMP proteins and that Wnt induction of ALP in C3H10T1/2 cells is dependent on the expression of BMPs.

**EXPERIMENTAL PROCEDURES**

Effects of Sclerostin, Other BMP Antagonists, and Wnt-3a on ALP Activity in Mouse Menenchymal C3H10T1/2 Cells—C3H10T1/2 cells (American Type Culture Collection, Manassas, VA) were plated in 96-well dishes (25,000 cells/well) in Dulbecco’s modified Eagle’s medium supplemented with high glucose and glutamine, 10% fetal calf serum, 1% penicillin/streptomycin, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 55 μM β-mercaptoethanol, and 20 mM HEPES (pH 7.3). Human sclerostin-FLAG protein (9–50 μg/ml), mouse noggin-FC, mouse gremlin, human BMP receptor 1A (BMPR1A)-FC, and human Dkk-1 were preincubated with 600 ng/ml BMP-6 or 100 ng/ml mouse Wnt3A in medium for 1 h prior to addition to cells. Cells were harvested 72 h later for assay of ALP activity (Pierce) by determining p-nitrophenylphosphate (pNp) production from the Wnt-3a reporter (determined in mg of protein/30 min of incubation at room temperature). Similar experiments were also conducted with conditioned medium collected from murine L1 cells stably transfected with a Wnt-3a expression vector (L Wnt-3A, American Type Culture Collection) (15). All recombinant BMP antagonists, Wnt proteins, and Dkk-1 were purchased from R&D Systems.

**Reverse Transcription-PCR—**C3H10T1/2 cells were plated in regular growth medium and treated with 600 ng/ml BMP-6 or 100 ng/ml Wnt3A. Cells were harvested at various time points for preparation of RNA. RNA was prepared using Strataprep Absolutely RNA mini-prep kits (Stratagene, San Diego, CA). cDNA synthesis was performed using 1 μg of total RNA, oligo(dT) primers, and the Superscript First Strand cDNA synthesis system according to the manufacturer’s directions (Invitrogen). PCR was performed using Platinum TaqDNA polymerase (Invitrogen), and the following PCR primers were used to detect the expression of the murine genes coding for ALP, BMP-4, and the housekeeping gene, defender against death (DAD) (3). Primer sets crossed intron/exon boundaries to eliminate amplification of genomic DNA or generation of larger size amplicons. All PCR products were

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‡ The abbreviations used are: BMP, bone morphogenetic protein; BMPR1A, BMP receptor 1A; ALP, alkaline phosphatase; DAD, defender against death; FC, immunoglobulinFc.
verified by sequencing. The primers are as follows: DAD (product size 250 nucleotides), sense, 5'-AGAGCGAAGGGAGTTCTCTGGACGTGCCACC-3' and antisense, 5'-TCCTCTTTGCCAGCACGATCCTG-3'; ALP (400 nucleotides), sense, 5'-CTTGCAAGGATCGGAACG-3' and antisense, 5'-GACCTGAGCGTTGGTGTTATATGT-3'; and BMP-4 (400 nucleotides), sense, 5'-CTGCCGCTGCAACCCAGCCT-3' and antisense, 5'-GCGCCTCCTAGCAGGACT-3'.

β-Catenin Accumulation Assay—Confluent cultures of murine L1 cells (American Type Culture Collection) (15) were treated with recombinant Wnt-3A (0.1 μg/ml), human sclerostin-FLAG (40 μg/ml), noggin-FC (5 μg/ml), BMP R1A-FC (40 μg/ml), and Dkk-1 (5 μg/ml). After 30 min, the plates were washed with ice-cold phosphate-buffered saline, and the cells were harvested with SDS-PAGE loading buffer. Protein concentrations were normalized, and Western blot analysis was performed with an anti-β-catenin antibody (Cell Signaling Technology, Beverly, MA).

RESULTS

Sclerostin and Other BMP Antagonists Block BMP-induced and Wnt-induced ALP Activity—To determine whether sclerostin affects the activity of Wnt proteins, we compared the effects of sclerostin on BMP- and Wnt-induced activity in murine C3H10T1/2 cells. BMP-6 stimulated ALP activity in C3H10T1/2 cells. As we have shown previously (3), human sclerostin-FLAG reduced this ALP activity in a dose-dependent manner (Fig. 1). Noggin-FC and gremlin also demonstrated blocking behavior (Fig. 1). ALP activity in the C3H10T1/2 cells was enhanced upon treatment of the cells with purified recombinant Wnt-3A protein or conditioned medium from murine L1 cells stably expressing Wnt-3A protein (data not shown). This Wnt-induced ALP activity was diminished with increasing concentrations of sclerostin, noggin-FC, and gremlin (Fig. 1).

BMP Receptor 1A (BMPR1A) Blocks Both BMP-induced and Wnt-induced ALP Activity, and Dkk-1 Blocks Wnt but Not BMP-induced ALP Activity—To investigate the underlying mechanism by which sclerostin decreased Wnt-induced ALP activity, we sought to disrupt ALP induction through the use of antagonists with restricted modes of action. This was accomplished through the use of soluble BMP receptor 1A (BMPR1A) and Dkk-1, a known inhibitor of the Wnt pathway (16). C3H10T1/2 cells were treated with a fixed amount of BMP-6 or Wnt-3A in the presence of increasing concentrations of BMPR1A or Dkk-1. The results are shown in Fig. 2. BMPR1A decreased BMP-6-induced ALP activity and Wnt3A-induced ALP activity in a dose-dependent manner (Fig. 2). In contrast, Dkk-1 effectively decreased Wnt-3A-induced ALP activity but had no effects on BMP-induced ALP activity (Fig. 2). Collectively, these data begin to delineate the interplay between the BMP and Wnt pathways.

Wnt-3A Induces mRNAs for BMP-4—Our observations that BMPR1A and other BMP antagonists were able to reduce Wnt-3A-induced ALP activity suggested that BMPs played a role in
the Wnt-3A-induction of ALP activity. To investigate this, C3H10T1/2 cells were treated with BMP-6 or Wnt-3A, and RNA was prepared and analyzed by reverse transcription-PCR for the presence of a select group of BMPs. As can be seen in Fig. 3, ALP mRNA levels were apparent in BMP-6-treated cells at the 6-h time point, whereas there was a 12-h delay in the Wnt-treated cells. In the latter, the increase in ALP mRNA levels is preceded by the appearance of mRNA for BMP-4, highlighting the differential timing of induction. The mRNA levels for BMP-4 and ALP in the Wnt samples peaked by 24 h and slowly decreased over the 72-h incubation period. The timing of Wnt-3A induction of BMP-4 and ALP is consistent with BMP-4 being a critical autocrine factor for the Wnt-induced ALP in C3H10T1/2 cells.

**Sclerostin Inhibition of Wnt-3A Activity Is Mediated by BMPs**

**FIG. 2.** BMP receptor 1A-FC fusion protein blocks both BMP-6- and Wnt-3A-induced ALP activity, whereas the Dkk-1 Wnt inhibitor blocks only Wnt-3A-induced ALP. Top two panels, BMPR1A-FC but not Dkk-1 block ALP of BMP-6-treated C3H10T1/2 cells. Bottom two panels, both BMPR1A-FC and Dkk-1 block ALP in Wnt-3A-treated C3H10T1/2 cells.

**FIG. 3.** Wnt-3A and BMP-6 treatment of C3H10T1/2 cells induces the expression of BMP-4, BMP-6, and alkaline phosphatase. Expression of ALP, BMP-4, and DAD (RNA loading control) is measured by reverse transcription-PCR. The time courses (time in hours along the top) of C3H10T1/2 cells treated with Wnt-3A (left panel) and BMP-6 (right panel) are shown. No DNA and cDNA PCR controls are on the right.

**FIG. 4.** Wnt-3A induction of β-catenin is blocked by DKK1 but not by BMP antagonists. Cells lysed after a 30-min treatment of mouse L1 cells with Wnt-3A. An anti-β-catenin Western blot is shown. Treatments are as labeled.
Sclerostin Inhibition of Wnt-3A Activity Is Mediated by BMPs

Sclerostin, BMP antagonists

BMPs

BMP receptor

DKK 1

WNT

LRP5

FRZ

Frizzled

ALP

β-catenin

FIG. 5. A model for sclerostin blockade of Wnt induction ALP in C3H10T1/2 cells. Wnt induces the expression of BMP proteins in an autocrine signaling cascade. BMP antagonists can then block the activity of expressed BMP proteins. Our data demonstrate that BMP protein expression is a necessary step in Wnt-3A-induced C3H10T1/2 expression of ALP. Our data show that the BMP expression and β-catenin accumulation are the results of Wnt activity. The Wnt-induced BMP expression may or may not be mediated by β-catenin. FRZ, Frizzled.

effects on Wnt-induced β-catenin levels (Fig. 4), supporting the hypothesis that these BMP antagonists do not directly affect immediate-early Wnt signaling.

DISCUSSION

There are many reports that describe the intertwined nature of BMP and Wnt signaling and their involvement in morphogenesis and cell differentiation (17–19). The recent description and characterization of the genes responsible for high bone mass syndromes (lrp-5 and sost) point to key roles for both Wnt and BMP pathways in osteoblast differentiation and maturation (2, 12). In our analysis, we found that sclerostin is an inhibitor of osteoblast differentiation induced by both BMP and Wnt pathways, suggesting that multiple antagonistic properties could be associated with this protein. This observation is not without precedent. Cerberus, a BMP antagonist with some similarity to sclerostin, was shown to bind to Wnt proteins as well as to nodal, suggesting that these BMP antagonists could be multifunctional inhibitors including inhibitors of Wnt proteins (20, 21). We have also shown that sclerostin is pleiotropic: blocking BMP activity as a BMP antagonist, but also binding to and neutralizing the activity of noggin, which results in the activation of BMP activity (22).

For these reasons, we postulated that sclerostin might be a direct inhibitor of Wnt activity. However, we found that this inhibition was indirect. Instead, sclerostin inhibited BMPs produced in an autocrine loop by Wnt-3A. Activation of the canonical Wnt signaling pathway can be measured by the accumulation of β-catenin in the presence of Wnt protein (13, 14). Sclerostin and other BMP antagonists had no effect on β-catenin accumulation, whereas Dkk-1, a Wnt inhibitor (16), was able to completely block Wnt-induced β-catenin accumulation. We tested for physical interaction between Wnt-3A and sclerostin by Biacore analysis and immunoprecipitation and found no evidence of direct interaction (data not shown). We also found that other BMP antagonists including noggin, gremlin, and the BMPR1A-FC fusion protein could also block Wnt-3A-induced ALP. Wnt-3A treatment of C3H10T1/2 cells strongly induced the expression of BMP-4 12 h before the appearance of ALP expression. From these data, we suggest that Wnt-3A induced the expression of BMPs, and it is this expression that is inhibited by the BMP antagonists (Fig. 5).

The Wnt-induced BMP expression may or may not be mediated by β-catenin.

In contrast to our results, Rawadi et al. (23) reported that Wnt-induced ALP expression in C3H10T1/2 cells was blocked by Wnt inhibitors but not by BMP inhibitors, and conversely, BMP-induced ALP was inhibited by the Wnt inhibitor, Dkk-1. From these data, the authors concluded that BMP-induced ALP was dependent on Wnt signaling, a direct contradiction to our findings (23). The reasons for the differences in conclusions are unknown. A major difference between our studies is that we have used recombinant purified sources for our growth factors and antagonists, whereas Rawadi et al. (23) measured effects after a combination of transfections and use of recombinant factors. It is entirely possible that these or other technical differences could lead to such different conclusions.

In summary, as would be expected from the high bone mass and the sclerosteosis phenotypes, both the Wnt and the BMP pathways regulate bone formation. In this study, sclerostin plays a role as master controller of both pathways, modulating both BMP and Wnt signaling by working as a BMP antagonist and demonstrates that BMPs are ultimate growth factors responsible for the commitment step for osteoblastic differentiation.

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