Sclerostin Is a Novel Secreted Osteoclast-derived Bone Morphogenetic Protein Antagonist with Unique Ligand Specificity*

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Sclerosteosis is a progressive sclerosing bone dysplasia. Sclerostin (the SOST gene) was originally identified as the sclerosteosis-causing gene. However, the physiological role of sclerostin remains to be elucidated. Sclerostin was intensely expressed in developing bones of mouse embryos. Punctuated expression of sclerostin was localized on the surfaces of both intramembranously forming skull bones and endochondrally forming long bones. Sclerostin-positive cells were identified as osteoclasts. Recombinant sclerostin protein produced in cultured cells was efficiently secreted as a monomer. We examined effects of sclerostin on the activity of BMP2, BMP4, BMP6, and BMP7 for mouse preosteoblastic MC3T3-E1 cells. Sclerostin inhibited the BMP6 and BMP4, BMP6, and BMP7 with high affinity but bound to BMP2 and BMP4 with lower affinity. In conclusion, sclerostin is a novel secreted osteoclast-derived BMP antagonist with unique ligand specificity. We suggest that sclerostin negatively regulates the formation of bone by repressing the differentiation and/or function of osteoclasts induced by BMPs. Since sclerostin expression is confined to the bone-resorbing osteoclast, it provides a mechanism whereby bone apposition is inhibited in the vicinity of resorption. Our findings indicate that sclerostin plays an important role in bone remodeling and links bone resorption and bone apposition.

Sclerosteosis is a progressive sclerosing bone dysplasia with an autosomal recessive mode of inheritance. Sclerosteosis is clinically and radiologically very similar to van Buchem disease (1, 2). By linkage analysis of families with these diseases, the disease-causing genes were mapped to the same chromosomal 17q12-q21 region, supporting the hypothesis that both diseases are caused by mutations in the same gene. By the positional cloning strategy, sclerostin (the SOST gene), which was mutated in sclerosteosis patients, was identified (1, 2). Sclerostin was found to be expressed in human long bones and cartilage using the polymerase chain reaction. However, the expression of sclerostin in the bones and cartilage was not examined in detail. The pathogenesis and genetics of sclerosteosis suggest that inhibition of sclerostin could lead to increased bone density. This definitely makes sclerostin and its pathway interesting targets for the development of anabolic agents against osteoporosis (1, 2). Sclerostin encodes a protein of 213 amino acids with a putative signal peptide for secretion, and sclerostin has six conserved cysteine residues and one conserved glycine residue that are essential to form a cystine knot. The spacing of cysteine residues is highly homologous to that of bone morphogenetic protein (BMP)³ antagonists of the DAN/cerberus family, indicating that sclerostin might be a BMP antagonist (1, 2). However, the biological activity of sclerostin is not known. Therefore, the physiological role of sclerostin and its mechanism of action remain to be elucidated.

We examined the expression of sclerostin in mouse embryonic bones by in situ hybridization and the biological activity of recombinant sclerostin protein. Sclerostin was found to be expressed in osteoclasts both in bones forming directly from mesenchyme and via a cartilage template. We showed that sclerostin encoded a novel secreted osteoclast-derived BMP antagonist with unique ligand specificity. The present findings indicate that sclerostin negatively regulates the formation of bone by repressing the differentiation and/or function of osteoclasts induced by BMPs. Sclerostin apparently links the functions of osteoclasts and osteoblasts in bone resorption and bone apposition, respectively, and it therefore provides a novel mechanism for the regulation of bone remodeling.

EXPERIMENTAL PROCEDURES

In Situ Hybridization—The embryonic mouse tissues (embryonic day 10 to newborn) were fixed in 4% paraformaldehyde, embedded in paraffin, and serially sectioned. Radioactive in situ hybridization procedures for tissue sections were carried out as described (3). Probes were labeled with [³²P]UTP; exposure time was 14 or 28 days. The preparation of the Bop and MMP-9 RNA probes have been previously described (4, 5). The sclerostin RNA probe was prepared using mouse sclerostin (SOST) cDNA (680 bp) as a template (2).

Production of Recombinant Mouse Sclerostin in Insect Cells—The mouse sclerostin cDNA with a 75-base pair DNA fragment encoding an E tag (GAPVPFYDPLEPR) and a His tag (HIHHHHH) at the 3’ terminus of the coding region was constructed in a transfer vector DNA, pBacPAK9. Recombinant baculovirus containing the sclerostin cDNA with the tag sequences was obtained by cotransfection of Sf9 cells with the recombinant pBacPAK9 and a BacPAK6. High Five cells infected with the recombinant baculovirus were cultured at 27 °C for 72 h in Grace’s insect cell culture medium (Invitrogen) containing 10% fetal bovine serum. Recombinant mouse sclerostin was purified from the culture medium by affinity chromatography using nickel-nitriol triacetic acid-agarose and desalted by gel filtration chromatography using Bio-Gel P-6 DG in PBS containing 50 μg/ml bovine serum albumin.

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The abbreviations used are: BMP, bone morphogenetic protein; E, embryonic day n.
Detection of Recombinant Mouse Sclerostin by Western Blotting Analysis—The culture medium and cell lysate of High Five cells infected with recombinant baculovirus were separated by SDS-12.5% polyacrylamide gel electrophoresis under reducing or nonreducing conditions and transferred onto a nitrocellulose membrane (Hybond-ECL). The protein with the E tag on the membrane was visualized as described (6). Purified recombinant mouse sclerostin protein (0.35 μg) was separated by SDS-12.5% polyacrylamide gel electrophoresis under reducing conditions and then stained with Coomassie Brilliant Blue R-250. Prestained Protein Marker Broad Range (New England Biolabs) was used as molecular mass standard proteins.

MC3T3-E1 Cell Culture—Mouse preosteoblastic MC3T3-E1 cells were maintained and subcultured for 3 or 4 days at 37 °C in a humidified atmosphere of 5% CO2 in air in α-modified minimum essential medium containing 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin in a humidified CO2 incubator.

Alkaline Phosphatase Activity in MC3T3-E1 Cells—For determination of alkaline phosphatase activity, MC3T3-E1 cells were plated at a density of 1 × 105 cells/well in 48-well plates. After the cells had reached confluence, the medium was replaced with a minimum essential medium containing 100 units/ml penicillin G, 100 μg/ml streptomycin, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid, and cells were cultured for 24 h. The cells were then cultured in a minimum essential medium containing 1% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid, and cells were cultured for 24 h. The cells were then cultured in a minimum essential medium containing 1% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid, and cells were cultured for 24 h. The cells were then cultured in a minimum essential medium containing 1% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid, and cells were cultured for 24 h. The cells were then cultured in a minimum essential medium containing 1% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid, and cells were cultured for 24 h. The cells were then cultured in a minimum essential medium containing 1% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid, and cells were cultured for 24 h. The cells were then cultured in a minimum essential medium containing 1% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid, and cells were cultured for 24 h. The cells were then cultured in a minimum essential medium containing 1% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid, and cells were cultured for 24 h. The cells were then cultured in a minimum essential medium containing 1% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid, and cells were cultured for 24 h. The cells were then cultured in a minimum essential medium containing 1% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid, and cells were cultured for 24 h. The cells were then cultured in a minimum essential medium containing 1% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid, and cells were cultured for 24 h.

Expression of Sclerostin during Bone Formation—Since sclerosteosis also affects bone modeling and remodeling in the skull (1, 2), we examined the expression of sclerostin mRNA in the developing skull of mouse embryos by in situ hybridization. Most skull bones develop directly from the mesenchyme by the mechanism of intramembranous bone formation. The initiation of many craniofacial bones is evident in embryonic day 13 (E13) mouse embryos. However, no sclerostin mRNA expression was seen in the head mesenchyme in frontal sections of E11–E13 embryos (Fig. 1A and data not shown). First cells expressing sclerostin mRNA were detected at E14 at the sites of osteogenesis (data not shown). Sclerostin mRNA expression increased at E15, and it appeared as bright punctuated expression in the developing mandibular and maxillary bones (Fig. 1B). The sclerostin mRNA-expressing cells were clearly localized on the surfaces of forming bones, but they covered only some parts of the developing bones. This is shown by the expression pattern of the osteoblast marker gene bone sialoprotein (Bsp), which covers the total area of bone formation (Fig. 1C). In newborn mice, sclerostin mRNA-expressing cells were located in all developing bones in the head. Isolated cells expressing sclerostin mRNA were present on the surfaces of the forming calvarial bones and in the palatal bone (Fig. 1D). Expression was particularly intense in the bone surrounding the growing tooth germs (Fig. 1D).

Since sclerosteosis also affects bone modeling and remodeling in the diaphyseal region of long bones (1, 2), we examined the expression of sclerostin mRNA in developing long bones and other endochondral bones of mouse embryos by in situ hybridization. In the tibia at E16, sclerostin mRNA was mainly detected in the perichondrium of the hypertrophic chondrocyte region and the peristeum of the diaphyseal region (Fig. 1F). In the tibia at E18, sclerostin mRNA was detected in the trabecular bone in addition to the perichondrium and periosteum. We also examined the expression of sclerostin mRNA in other endochondrally developing bones including the radius and iliac bone. The expression profile in each of these bones was essentially similar to that in the tibia (data not shown). In addition, sclerostin mRNA-expressing cells were located around the rib cartilage (Fig. 1F).

Sclerostin mRNA expression was largely restricted to the bones, and most other tissues in the sections of E11–E13 whole embryos were negative. Strong expression was found in the endothelium of the pharyngeal artery in E11 embryos (Fig. 1A). In addition, expression was seen in the liver of E12 embryos, and it was localized to islands of hematopoietic cells (Fig. 1G).

Coexpression of Sclerostin with MMP-9—Although the sclerostin mRNA-expressing cells were colocalized with osteoblasts on the surfaces of forming bones, their distribution was restricted only to some areas of bone formation. Sclerostin mRNA expression was also clearly punctuated, and it appeared to localize to isolated large cells. This distribution resembled that of bone-resorbing osteoclasts. The matrix metalloproteinase MMP-9 has been shown to be a marker of osteoclasts, in particular in the developing bones (4, 8). Comparison of the patterns of MMP-9 mRNA and sclerostin mRNA-expressing cells in the intramembranously forming bones in the embryonic
Sclerostin is a member of the cystine knot family. The spacing of cysteine residues in sclerostin is highly homologous to that of BMP antagonists, DAN, cerberus and gremlin, of the DAN/cerberus family, indicating that sclerostin might be a BMP antagonist (1, 2). BMPs were originally identified as proteins in bone that induce ectopic bone and cartilage formation in vivo. BMPs are signaling molecules for the stimulation of osteoblast differentiation (10, 11). We examined effects of sclerostin on the activity of BMP2, BMP4, BMP6, and BMP7 for the differentiation of MC3T3-E1 cells by determining alkaline phosphatase activity, a marker for osteoblast differentiation (12). BMP2, BMP4, BMP6, and BMP7 greatly stimulated the alkaline phosphatase activity in MC3T3-E1 cells (Fig. 4). We examined effects of recombinant sclerostin protein on the BMP activity. The activity of BMP6 was inhibited by sclerostin in a dose-dependent manner (Fig. 4A). The activity of BMP7 was weakly but significantly inhibited by sclerostin (Fig. 4B). In contrast, the activity of BMP2 and BMP4 was not inhibited by sclerostin (Fig. 4, C and D). Noggin, a BMP antagonist, is known to antagonize BMP2, BMP4, and BMP7, with a higher activity for BMP2 and BMP4 (13). We also examined the effect of recombinant mouse noggin protein on the BMP activity. In contrast to sclerostin, noggin significantly antagonized the activity of BMP2, BMP4, BMP6, and BMP7, with a higher activity for BMP2 and BMP4 (Fig. 4).

Binding of Sclerostin to BMPs—Since sclerostin significantly inhibited the activity of BMP6, we examined the binding of sclerostin to BMP6 using the BIACore system with the recombinant sclerostin protein-fixed sensor tip. Typical binding and dissociation curves of BMP6 were obtained (Fig. 5). The equilibrium dissociation constant was also determined. Sclerostin was found to bind to BMP6 with high affinity (Table I). We also examined the binding of sclerostin to BMP2, BMP4, and BMP7. Although typical binding and dissociation curves were also obtained (data not shown), sclerostin was found to bind to BMP7 with lower affinity and to BMP2 and BMP4 with much lower affinity (Table I).

**DISCUSSION**

Sclerosteosis is a progressive sclerosing bone dysplasia characterized by massive bone overgrowth with an autosomal recessive mode of inheritance. The disorder affects bone modeling and remodeling, especially in the skull and diaphyseal region of long bones (1, 2). Elevated alkaline phosphatase activity and increased rates of bone formation have suggested a defect in osteoblast function. Sclerostin (the SOST gene) was originally identified by positional cloning of the disease-causing gene in sclerosteosis. Therefore, inhibition of sclerostin was expected to lead to increased bone density, definitely making sclerostin and its pathway interesting targets for the development of anabolic...
agents against osteoporosis (1, 2). However, the physiological role of sclerostin and its mechanism of action remain to be elucidated.

Bones are formed through two distinct developmental processes, intramembranous ossification and endochondral ossification (14, 15). Formation of most skull bones is achieved by intramembranous ossification, whereas the rest of the skeleton including long bones and vertebrae develop through the process of endochondral ossification in which cartilage is converted into bone. Since both skull bones and long bones are affected in the sclerosteosis patients, sclerostin is apparently involved in both intramembranous and endochondral bone formation (1, 2).

**Sclerostin** was expressed on the surfaces of forming intramembranous bones in the embryonic mouse head. The intramembranous bones are initiated as condensates of mesenchymal cells. The cells differentiate directly to osteoblasts and secrete the extracellular matrix of bone, which subsequently mineralizes. The advancing development is characterized by the process of bone remodeling where the shaping of the bone is regulated by localized bone resorption by osteoclasts and apposition by osteoblasts.

As in the skull bones, **sclerostin** was also expressed in the long bones. Cartilage is formed by condensation of mesenchymal cells. Growth plate chondrocytes are arranged into columns that develop sequentially. Distal hypertrophic chondrocytes undergo apoptosis, and the cartilage is replaced by trabecular bone. In a separate process, cortical bone is generated via an intramembranous mechanism by osteoblasts derived from osteoprogenitor cells in the perichondrium (14, 15). **Sclerostin** is expressed in the perichondrium of the hypertrophic chondrocyte region as well as the periosteum and trabecular bone of the diaphyseal region. This indicated that **sclerostin** was expressed at the sites of bone formation in developing long bones.

**Sclerostin** was not expressed in any regions of the cartilaginous primordium except for the perichondrium of the hypertrophic chondrocyte region. These observations indicate that **sclerostin** plays a role in osteogenesis but not in chondrogenesis in the development of long bones. Sclerostin was also absent from the cartilaginous tissue in the head as well as ribs.

Osteoclasts are multinucleated cells differentiating from the hematopoietic precursors, and they express a variety of enzymes degrading the bone matrix and dissolving the mineral. Interestingly, the expression of **sclerostin** was confined to the osteoclasts but not to osteoblasts. We identified the osteoclasts in forming craniofacial bones by the detection of the expression of **MMP-9**, a matrix metalloproteinase that is specifically expressed in osteoclasts (4). A traditional marker for osteoclasts is TRAP, the tartrate-resistant acid phosphatase, which is intensely expressed by the active osteoclasts resorbing mineralized bone. Although **MMP-9** and TRAP expression largely overlap in osteoclasts, their expression depends on the developmental stage of bone (8). Whereas the osteoclasts resorbing mature bone are mostly TRAP-positive but MMP-9-negative, the osteoclasts degrading immature and nonmineralized bone

![Fig. 4. Effects of sclerostin and noggin on alkaline phosphatase activity in MC3T3-E1 cells induced by BMPs. MC3T3-E1 cells were treated with BMP6 (10 ng/ml) (A), BMP7 (25 ng/ml) (B), BMP2 (25 ng/ml) (C), or BMP4 (10 ng/ml) (D) and different concentrations of mouse recombinant sclerostin or 100 ng/ml noggin for 72 h. After treatment, alkaline phosphatase activity in MC3T3-E1 cells was determined. Results are the means \pm S.D. for five independent wells.](image)

![Fig. 5. Binding of sclerostin to BMP6. Mouse recombinant sclerostin was fixed on the carboxymethyl sensor tip. The binding of different concentrations of BMP6 on the tip was analyzed using the BIAcore 2000 system.](image)

**TABLE I**

|       | Kd (M) | ka (M⁻¹s⁻¹) | kₐ (s⁻¹) |
|-------|--------|-------------|---------|
| BMP6  | 4.72 × 10⁻³ | 8.27 × 10⁴  | 5.71 × 10⁻⁸ |
| BMP7  | 2.61 × 10⁻³ | 2.98 × 10⁴  | 8.76 × 10⁻⁸ |
| BMP2  | 2.54 × 10⁻³ | 2.53 × 10⁴  | 10.0 × 10⁻⁸ |
| BMP4  | 4.62 × 10⁻³ | 2.06 × 10⁵  | 22.4 × 10⁻⁸ |
during early bone development are MMP-9-positive and mostly TRAP-negative. Since sclerostin was expressed very early during intramembranous bone development, we compared its expression with MMP-9 and showed that the two genes were apparently localized to the same cells. We therefore conclude that sclerostin is expressed in osteoclasts. Interestingly, intense sclerostin expression was seen in the embryonic liver in hematopoietic cells, which are progenitors of osteoclasts.

Since sclerostin has a putative signal sequence at its amino terminus, it is expected to be a secreted protein (1, 2). We expressed mouse sclerostin in cultured cells. Recombinant sclerostin protein was efficiently secreted. Sclerostin has six conserved cysteine residues and one conserved glycine residue that are essential to form the cystine knot (1, 2). Most cystine knot proteins are secreted as dimers (16). The spacing of cysteine residues in sclerostin is highly homologous to that of BMP antagonists of the DAN/cerberus family. Cerberus and DAN, members of the DAN/cerberus family, are also secreted as homodimers (17, 18). They have an additional cysteine residue that is potentially used for dimerization. However, sclerostin does not have this cysteine residue. Recombinant sclerostin was found to be secreted as a monomer.

The similarity of the spacing of cysteine residues in sclerostin to that in the DAN/cerberus family also indicates that sclerostin might be a BMP antagonist (1, 2). BMPs are multifunctional molecules involved in morphogenesis during development. BMPs are also local signaling molecules for the stimulation of osteoblast differentiation (10, 11). BMPs are known to stimulate alkaline phosphatase activity, a marker for osteoblast differentiation, in mouse preosteoblastic MC3T3-E1 cells (12). The BMP family members can be divided into subgroups based on their structural similarities. BMP2 and BMP4 form one group, BMP5, BMP6, BMP7, and BMP8 form another group (19). Therefore, we examined the effects of sclerostin on the activity of BMP2, BMP4, BMP6, and BMP7 for the differentiation of MC3T3-E1 cells by determining the alkaline phosphatase activity of BMP2 and BMP7 and essentially none for BMP2 and BMP4. We also examined the binding of sclerostin to these BMPs using the BIAcore system. Sclerostin was found to bind to BMP2 with much higher affinity. In contrast, sclerostin bound to BMP4 with lower affinity and to BMP2 and BMP4 with much lower affinity. These results were essentially consistent with those of the inhibition experiments. Therefore, sclerostin appears to act as a BMP antagonist by binding extracellularly to BMP.

Noggin, a BMP antagonist, was originally isolated from Xenopus laevis based upon an ability to rescue dorsal development in embryos ventralized by UV treatment. Noggin is known to bind and antagonize BMP2, BMP4, and BMP7, with a higher affinity for BMP2 and BMP4 (13). Our results also indicate that noggin can significantly antagonize BMP2 and BMP4 and more weakly antagonize BMP6 and BMP7. Chordin, a BMP antagonist, plays major roles in the formation of the dorsoventral axis. Chordin also binds BMP2, BMP4, and BMP7 in a way similar to noggin (20). DAN, cerberus, and gremlin, members of the DAN/cerberus family, are also known to bind and antagonize BMP2, BMP4, and BMP7 (21–23). These results indicate that sclerostin is a novel BMP antagonist with unique ligand specificity.

BMP6−/− mice were viable and fertile, but the examination of skeletogenesis in late gestation embryos revealed a consistent delay in ossification strictly confined to the developing sternum (24). In situ hybridization studies have indicated that BMP6 is expressed in overlapping patterns with other BMP genes in developing bones. Hence, it is possible that other BMPs may functionally compensate for BMP6 in the null mice (24).

In conclusion, sclerostin is a novel secreted BMP antagonist with unique ligand specificity, and its expression is confined to osteoclasts in both endochondral and intramembranous bones. To our knowledge, sclerostin is the first BMP antagonist that is localized in osteoclasts. We suggest that sclerostin plays a specific role in bone development and that it negatively regulates the formation of bone by repressing the BMP-induced differentiation and/or function of osteoblasts. Sclerostin appears to provide a mechanism whereby bone apposition is prevented locally in the vicinity of bone resorption. Sclerostin thereby links the processes of resorption and apposition via a previously unknown mechanism. The importance of communication between osteoblasts and osteoclasts is known to have an important role in the regulation of resorption, as exemplified by the regulation of osteoclast differentiation by the RANK ligand OPGL (a tumor necrosis family cytokine) expressed by osteoblasts (25). The present findings provide a novel insight into osteogenesis and in particular into the interaction of osteoclast and osteoblast functions during bone remodeling.

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