Production and Characterization of Monoclonal Antibodies to Human Sclerostin

Theodore A. Craig,¹ Stacy L. Sommer,¹ Thomas G. Beito,² and Rajiv Kumar^{1,3}

We developed and characterized monoclonal antibodies directed against the amino-terminal and carboxy-terminal regions of human and mouse sclerostin (scl). Amino-terminal and carboxy-terminal scl peptides with limited homology to scl domain-containing protein-1 were synthesized using f-moc chemistry. The peptides were conjugated to keyhole limpet hemocyanin and the conjugates were used for immunization of mice. Monoclonal antibodies were obtained and characterized using bacterially expressed and insect cell-expressed recombinant scl. The amino-terminal (IgG 2aK) and carboxy-terminal (IgG 2bK) antibodies bound bioactive sclerostin that was expressed in an insect-cell expression system with dissociation constants in the nanomolar range. The antibodies are potentially useful agents that can be used for modulating sclerostin bioactivity.

Introduction

SCLEROSTIN (SCL) IS A SECRETED PROTEIN that inhibits bone formation.^(1–7) Sclerostin gene (SOST) mutations, either in the promoter or coding sequence of the gene, are present in van Buchem disease or sclerosteosis, and result in a general progressive overgrowth and sclerosis of the skeleton^(8–11) due to reduction of full-length scl expression or expression of mutant scl with reduced activity. Scl antagonizes bone morphogenic protein (BMP) and canonical Wnt signaling.^(2–5,12–14) Scl antagonizes bone formation by binding to the first β -propeller of low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6), which function as Wnt co-receptors.⁽¹⁵⁾ High bone mass in sclerosteosis and van Buchem disease results from increased Wnt signaling due to the reduction of scl concentrations or reduced activity of mutant scl.^(16–19) SOST mRNA has been detected in aortic tissue and in the otic vesicle (epithelium) and odontoblasts, in addition to osteoblasts and osteocytes of bone, suggesting that scl may have functions other than the regulation bone density.⁽¹²⁾ Since sclerostin is important in modulating bone formation, antibodies directed against this protein could be used to increase bone formation and treat low bone mass syndromes such as osteoporosis.

We report on the development and properties of region-specific antibodies directed against human and mouse sclerostin. We demonstrate that the antibodies bind to bioactive sclerostin, with dissociation constants in the nanomolar range.

Materials and Methods

Protein concentrations were determined using the Bio-Rad Protein Reagent (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as standard and by UV absorbance. Protein sequencing was carried out as described previously.^(20–22) DNA sequencing and oligonucleotide synthesis were performed using an automated DNA sequencer and an oligonucleotide synthesizer, respectively (Applied Biosystems, Foster City, CA).^(23–25) SDS-PAGE was carried out using a PhastGel apparatus (GE Healthcare, Piscataway, NJ). Reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Production of monoclonal antibodies to sclerostin

Scl peptides with limited homology to scl domain-containing protein-1 (GenBank accession no. NM-015464) were synthesized using f-moc chemistry.^(26,27) Sequences were as follows: *scl mouse peptide* 61-78, 61-GRPPHHPYDAK DVSEYSC-78 (human and mouse sequences are not identical but are similar); and *scl human peptide* 168-183 plus an extra cysteine for KLH conjugation, 168-KRLTRFHNQSELKDFG-183 + C (human and mouse sequences are identical).

Monoclonal antibodies were generated using standard methods.⁽²⁸⁾ Hybridoma supernatants were initially screened in 96-well plates for scl antibodies by ELISA assays using bacterially expressed human 24-213 sclerostin-maltose binding protein as antigen (expressed as noted below), and anti-mouse IgG-alkaline phosphatase for detection.

¹Nephrology Research, Department of Medicine; ²Department of Immunology; ³Biochemistry and Molecular Biology; Mayo Clinic College of Medicine, Mayo Clinic, Rochester, Minnesota.

Following cloning, production of antibodies by positive clones was verified by SDS-PAGE multichannel immunoblotting. Isotyping of scl MAbs was carried out using the IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche Diagnostics).

Bacterial expression of sclerostin

For screening of clones expressing anti-scl monoclonal antibodies, the secreted form of human scl, amino acids 24-213, was expressed in the pMAL-P4E vector in *Escherichia coli* BL21 cells. The following 5' and 3' oligonucleotides were synthesized with *Bam*HI and *Sal*I restriction endonuclease sites to generate a PCR product using scl human SOST cDNA (GenBank accession no. AY358627) as template. The sequence of the 5' oligonucleotide is: 5'GAGAGGATCCCTGGAAGTTC TGTTCAGGGGCCCCAGGGGTGGCAGGCGTTCAAGA ATGATGCCACG3' (single-underscored nucleotides represent the *Bam*HI site and double-underscored nucleotides are those of the PreScission protease site). The sequence of the 3' oligonucleotide is: 5'GAGAGTCGACCTAGTAGGCGTTCTC CAGCTCGGCCTGGTTG3' (the underscored nucleotides represent the *Sal*I restriction endonuclease site).

The PCR product was cut with the appropriate restriction endonucleases, purified, and cloned into *Bam*HI and *Sal*I restriction endonuclease cleaved pMAL-P4E (New England Biolabs, Beverly, MA). Expression in *E. coli* cultures growing at 37°C 250 rpm in a reciprocal shaking incubator was induced at 37°C with 1 mM IPTG for 4 h. Expressed full-length 24-213 human sclerostin-maltose binding protein secreted into the extracellular periplasmic space was obtained by osmotic shock, and was purified on an amylose resin. Sclerostin-MBP was used to screen potential hybridoma clones by immunoblot analyses for expression of the monoclonal antibodies.

Sclerostin production in Trichoplusia ni cells

To express the secreted form of human scl, amino acids 24-213, a 5' oligonucleotide with a 5' Kozak consensus sequence, a melittin secretory signal sequence and a *Bam*HI site, and a 3' oligonucleotide without a stop codon were used to generate a PCR product using human SOST cDNA (GenBank accession no. AY358627) as template.⁽²⁹⁾ The sequence of the 5' oligonucleotide is: 5'GAGAGGATCCGACATGGGGATGAAAT TCTTAGTCAACGTTGCCCTTGTTTTATGGTCGTATACA TTTCTTACATCTATGCCCAGGGGTGGCAGGCGTTCAAG AATGATGCCACG3'

The sequence of the 3' primer that contains an *Xho*I site and an enterokinase cleavage site is: 5'GAGACTCGAGGC CTTGTCATCATCGTCGTAGGCGTTCTCCAGCTCGGCCT GGTG3'

The PCR product was ligated into the pIB/V5-His vector (Invitrogen). Chimeric plasmid was used to transfect BTI-TN-5B1-4 High Five (*Trichoplusia ni*) cells multiple times using Cellfectin (Invitrogen). Stably transfected cells were selected with Blasticidin S-containing medium (100 µg/mL; InvivoGen, San Diego, CA) at 27°C. The presence of scl in the supernatant was detected with anti-V5-HRP-conjugated antibody and chemiluminescent methods.^(26,29) Stably transformed cells (0.3×10^6 cell/mL) were grown for ~7 days after inoculation in suspension cultures (20 2-L flasks containing 0.6 L

Express Five SFM medium/2-L flask) at 27°C with shaking at 120 rpm. Cell-free medium was filtered and applied overnight at 4°C to 20 mL SP Sepharose resin (GE-Amersham, Piscataway, NJ). The SP Sepharose was washed 6 times with 10 column volumes 20 mM Na₂HPO₄ (pH 7.0). Protein was eluted using 4 column volumes of 1 M NaCl, 20 mM Na₂HPO₄ (pH 7.0). The medium was applied to 20 mL Ni Fast Flow Sepharose 6 (GE-Amersham). The resin was washed with 1.5 L 1 M NaCl, 20 mM Na₂HPO₄ (pH 7.0) buffer. Adsorbed protein was eluted with 40 mL 1 M imidazole, 1 M NaCl, 20 mM Na₂HPO₄ (pH 7.0), dialyzed against 1 M NaCl, 20 mM Na₂HPO₄ (pH 7.0), and loaded onto an analytical 5 mL HisTrap HP column (GE-Amersham). Proteins were eluted with a gradient of 0–1 M imidazole in 1 M NaCl, 20 mM Na₂HPO₄ (pH 7.0). Eluting proteins were analyzed by SDS/PAGE. PVDF immunoblot analyses were carried out using anti-V5 HRP (Invitrogen) or anti-scl monoclonal antibodies to identify fractions containing pure human 24-213 scl. Pooled fractions of high-purity scl were dialyzed against 150 mM Na₂HPO₄ (pH 7.0), filtered and loaded onto a 1 mL Mono S HR5/10 column. After elution using a gradient of 0–1 M NaCl in 150 mM Na₂HPO₄ (pH 7.0), fractions were analyzed as above, and scl fractions of highest purity were combined. Final protein was >95% pure.

Assessment of sclerostin bioactivity in human osteoblasts

hFOB cells⁽³⁰⁾ were transfected with M50 Super 8x TOP-Flash plasmid (Addgene, Cambridge, MA)⁽³¹⁾ using Lipofectamine (Invitrogen). hFOB medium contained vehicle, Wnt3a (10 ng/mL, R&D Systems, Minneapolis, MN), or a combination of Wnt3a (10 ng/mL) plus purified insect cell-produced scl (10 ng/mL). Twenty-four hours later, luciferase activity in cells was assayed using the Luciferase Reporter Assay kit (Promega, Madison, WI).

Determination of sclerostin MAb affinity using surface plasmon resonance spectroscopy

Recombinant insect cell-produced human sclerostin (~1200 RU) was coupled to a CM5 Biacore chip using amine coupling (Amine Coupling Kit, Biacore Life Sciences, GE Healthcare). Human recombinant sclerostin was diluted in Biacore buffer (10 mM sodium acetate buffer [pH 5.0]) to 30 µg/mL prior to coupling to a CM5 Biacore chip (channel 2 or 4 with channel 1 or 3 coupled without protein to serve as reference). Monoclonal antibodies were diluted into Biacore HBS-EP buffer (10 mM Hepes, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20 [pH 7.4]) to give concentrations between 500 and 0.05 nM (using a molecular mass of 150,000 Da for MAbs) and used for sensorgram experiments. A sensorgram control signal from a separate Biacore channel (channel 1, activated and quenched just as the experimental channels using amine coupling) was subtracted from the signal obtained from the monoclonal antibody. After multiple initial sensorgram runs to assure reproducibility, a series of sensorgram experiments were performed. For sensorgram analysis, flow rate was typically 30 mL/min. Chips were regenerated after each sensorgram run with 15–30 µL additions of 1 M NaCl in running buffer followed by 6–12 s additions of

FIG. 1. (A) SDS-PAGE of scl. Left and middle lanes, scl after nickel-affinity chromatography. Coomassie (left) or silver (middle) stain; right lane, scl after final Mono S chromatography (silver stain). (B) Immunostaining of scl with anti-scl MAb. Left lane, scl immunostaining with MAb directed against scl NH₂-terminal peptide; middle lane, scl immunostaining after nickel-affinity chromatography with MAb directed against scl NH₂-terminal peptide; right lane, scl immunostaining after nickel-affinity chromatography with MAb directed against scl COOH-terminal peptide.

10 mM glycine (pH 2.5) at a flow rate of 30 μ L/min. Data were analyzed using Biacore 3000 Control Software, version 3.2.

Sensorgram runs with 1200 RU sclerostin bound to a CM5 chip were performed with 0, 0.2, 0.5, 1.0, 2, and 5 nM of the MAb to the amino-terminus of scl, and with 0, 0.1, 0.2, 0.5, 1.0, 2, and 5 nM of the MAb to the carboxyl-terminus of scl in HBS-EP. The sensorgram for each concentration was run in duplicate. Each of the sensorgram results series was analyzed together, as a unit, using Biacore software for each MAb.

Results and Discussion

Characterization of bacterially expressed sclerostin

Bacterially expressed scl-MBP was purified with amylose affinity resins, served as an antigen for hybridoma supernatant screening in 96-well ELISA plates, and also was the antigen used for immunoblotting and verification of MAb producing clones.

Characterization of insect cell-expressed sclerostin

Scl was efficiently expressed in *Trichoplusia ni* cells (Fig. 1). Following Mono S chromatography, a single band was observed on SDS-PAGE using both Coomassie and silver staining methods (Fig. 1A). Amino-terminal sequencing of Mono S purified protein by Edman degradation showed two amino-terminal sequences, possibly as a result of two separate cleavage sites ($\sim$ 1:1). The NH₂-terminus showed protein beginning with glycine 25 (25-GWQAFKN-31), rather than the predicted glutamine 24, with a second sequence beginning at phenylalanine 29 (29-FKNDAT-35). Analysis of the trypsin-treated expressed scl by liquid chromatography-ESI-MS showed the appropriate tryptic peptides covering more than 55% of the expected sequences from across the entire molecule. Figure 2 shows that scl acts as a Wnt antagonist. At a concentration of 10 ng/mL, scl completely inhibited Wnt3a-enhanced activity in human osteoblast cells transfected with

FIG. 2. Effect of recombinant scl on Wnt3a mediated activity in human osteoblast cells. The addition of scl inhibited Wnt3a-mediated TOPFLASH luciferase activity. * $p < 0.05$.

the 8xTOPflash LEF/TCF luciferase reporter plasmid. This indicates that our preparation of scl is bioactive and is capable of antagonizing Wnt activity in bone.

Characterization of MAb against sclerostin

The MAb directed against the NH₂-terminal scl sequence detected the protein in the medium and, after purification, by nickel-affinity chromatography (Fig. 1B, left and middle lanes). Antibody isotyping showed that the antibody directed against the NH₂-terminal region of the protein had an IgG2aK isotype. The MAb directed against the C-terminal scl sequence recognized purified protein after nickel-affinity chromatography (Fig. 1B, right lane). Antibody isotyping showed that the antibody directed against the COOH-terminal region of the protein had an IgG2bK isotype. Both antibodies recognized a single band at the same M_r as that observed on Coomassie and silver staining. Sensorgram series using HBS-EP exhibited a K_D of $\sim 3 \times 10^{-9}$ M for the amino-terminal antibody and a K_D of $\sim 8 \times 10^{-9}$ M for the carboxyl-terminal antibody for the bound sclerostin.

In summary, our data describe the efficient production of MAbs raised against specific mouse and human peptides of the scl protein that recognize the full-length protein and neutralize its effects in osteoblast cultures. This should allow the use of scl MAbs for *in vivo* experiments in efforts to increase bone density.

Acknowledgments

This work was supported by NIH grants nos. DK 65830 and DK 76829 (to RK).

References

- ten Dijke P, Krause C, de Gorter DJ, Lowik CW, and van Bezooijen RL: Osteocyte-derived sclerostin inhibits bone formation: its role in bone morphogenetic protein and Wnt signaling. *J Bone Joint Surg Am* 2008;90(Suppl 1):31-35.

2. van Bezooijen RL, Roelen BA, Visser A, van der Wee-Pals L, de Wilt E, Karperien M, Hamersma H, Papapoulos SE, ten Dijke P, and Lowik CW: Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist. *J Exp Med* 2004;199:805–814.
3. van Bezooijen RL, Svensson JP, Eefting D, Visser A, van der Horst G, Karperien M, Quax PH, Vrieling H, Papapoulos SE, ten Dijke P, and Lowik CW: Wnt but not BMP signaling is involved in the inhibitory action of sclerostin on BMP-stimulated bone formation. *J Bone Miner Res* 2007;22:19–28.
4. Winkler DG, Sutherland MK, Geoghegan JC, Yu C, Hayes T, Skonier JE, Shpektor D, Jonas M, Kovacevich BR, Staehling-Hampton K, Appleby M, Brunkow ME, and Latham JA: Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. *Embo J* 2003;22:6267–6276.
5. Winkler DG, Sutherland MS, Ojala E, Turcott E, Geoghegan JC, Shpektor D, Skonier JE, Yu C, and Latham JA: Sclerostin inhibition of Wnt-3a-induced C3H10T1/2 cell differentiation is indirect and mediated by bone morphogenetic proteins. *J Biol Chem* 2005;280:2498–2502.
6. Winkler DG, Yu C, Geoghegan JC, Ojala EW, Skonier JE, Shpektor D, Sutherland MK, and Latham JA: Noggin and sclerostin bone morphogenetic protein antagonists form a mutually inhibitory complex. *J Biol Chem* 2004;279:36293–36298.
7. Yavropoulou MP, and Yovos JG: The role of the Wnt signaling pathway in osteoblast commitment and differentiation. *Hormones* 2007;6:279–294.
8. Loots GG, Kneissel M, Keller H, Baptist M, Chang J, Collette NM, Ovcharenko D, Plajzer-Frick I, and Rubin EM: Genomic deletion of a long-range bone enhancer misregulates sclerostin in Van Buchem disease. *Genome Res* 2005;15:928–935.
9. Balemans W, Ebeling M, Patel N, Van Hul E, Olson P, Dioszegi M, Lacza C, Wuyts W, Van Den Ende J, Willems P, et al: Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). *Hum Mol Genet* 2001;10:537–543.
10. Balemans W, Patel N, Ebeling M, Van Hul E, Wuyts W, Lacza C, Dioszegi M, Dikkers FG, Hildering P, Willems PJ, Verheij J, Lindpaintner K, Vickery B, Foernzler D, and Van Hul W: Identification of a 52 kb deletion downstream of the SOST gene in patients with van Buchem disease. *J Med Genet* 2002;39:91–97.
11. Brunkow ME, Gardner JC, Van Ness J, Paepers BW, Kovacevich BR, Prohl S, Skonier JE, Zhao L, Sabo PJ, Fu Y, Alisch RS, and Gillett L: Bone dysplasia sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. *Am J Hum Genet* 2001;68:577–589.
12. Kusu N, Laurikkala J, Imanishi M, Usui H, Konishi M, Miyake A, Thesleff I, and Itoh N: Sclerostin is a novel secreted osteoclast-derived bone morphogenetic protein antagonist with unique ligand specificity. *J Biol Chem* 2003;278:24113–24117.
13. Lowik CW, and van Bezooijen RL: Wnt signaling is involved in the inhibitory action of sclerostin on BMP-stimulated bone formation. *J Musculoskelet Neuronal Interact* 2006;6:357.
14. Li X, Zhang Y, Kang H, Liu W, Liu P, Zhang J, Harris SE, and Wu D: Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. *J Biol Chem* 2005;280:19883–19887.
15. Ellies DL, Viviano B, McCarthy J, Rey JP, Itasaki N, Saunders S, and Krumlauf R: Bone density ligand, Sclerostin, directly interacts with LRP5 but not LRP5G171V to modulate Wnt activity. *J Bone Miner Res* 2006;21:1738–1749.
16. O'Brien CA, Plotkin LI, Galli C, Goellner JJ, Gortazar AR, Allen MR, Robling AG, Bouxsein M, Schipani E, Turner CH, Jilka RL, Weinstein RS, Manolagas SC, and Bellido T: Control of bone mass and remodeling by PTH receptor signaling in osteocytes. *PLoS One* 2008;3:e2942.
17. Li X, Ominsky MS, Niu QT, Sun N, Daugherty B, D'Agostin D, Kurahara C, Gao Y, Cao J, Gong J, Asuncion F, Barrero M, Warming K, and Dwyer D: Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. *J Bone Miner Res* 2008;23:860–869.
18. Baron R, and Rawadi G: Targeting the Wnt/beta-catenin pathway to regulate bone formation in the adult skeleton. *Endocrinology* 2007;148:2635–2643.
19. van Bezooijen RL, ten Dijke P, Papapoulos SE, and Lowik CW: SOST/sclerostin, an osteocyte-derived negative regulator of bone formation. *Cytokine Growth Factor Rev* 2005;16:319–327.
20. Craig TA, Benson LM, Bergen HR 3rd, Venyaminov SY, Salisbury JL, Ryan ZC, Thompson JR, Sperry J, Gross ML, and Kumar R: Metal-binding properties of human centrin-2 determined by micro-electrospray ionization mass spectrometry and UV spectroscopy. *J Am Soc Mass Spectrom* 2006;17:1158–1171.
21. Craig TA, Benson LM, Venyaminov SY, Klimtchuk ES, Bajzer Z, Prendergast FG, Naylor S, and Kumar R: The metal-binding properties of DREAM: evidence for calcium-mediated changes in DREAM structure. *J Biol Chem* 2002;277:10955–10966.
22. Veenstra TD, Tomlinson AJ, Benson L, Kumar R, and Naylor S: Low temperature aqueous electrospray ionization mass spectrometry of noncovalent complexes. *J Am Soc Mass Spectrom* 1998;9:580–584.
23. Sanger F, Nicklen S, and Coulson AR: DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 1977;74:5463–5467.
24. Caruthers MH, Barone AD, Beaucage SL, Dodds DR, Fisher EF, McBride LJ, Matteucci M, Stabinsky Z, and Tang JY: Chemical synthesis of deoxyoligonucleotides by the phosphoramidite method. *Methods Enzymol* 1987;154:287–313.
25. Caruthers MH, Beaucage SL, Becker C, Efcavitch JW, Fisher EF, Galluppi G, Goldman R, deHaseth P, Matteucci M, McBride L, et al: Deoxyoligonucleotide synthesis via the phosphoramidite method. *Gene Amplif Anal* 1983;3:1–26.
26. Berndt TJ, Craig TA, McCormick DJ, Lanske B, Sitara D, Razzaque MS, Pragnell M, Bowe AE, O'Brien SP, Schiavi SC, and Kumar R: Biological activity of FGF-23 fragments. *Pflugers Arch* 2007;454:615–623.
27. Poduslo JF, Curran GL, Peterson JA, McCormick DJ, Fauq AH, Khan MA, and Wengenack TM: Design and chemical synthesis of a magnetic resonance contrast agent with enhanced in vitro binding, high blood-brain barrier permeability, and in vivo targeting to Alzheimer's disease amyloid plaques. *Biochemistry* 2004;43:6064–6075.
28. Ezzat MK, Howell KG, Bahler CK, Beito TG, Loewen N, Poeschla EM, and Fautsch MP: Characterization of

- monoclonal antibodies against the glaucoma-associated protein myocilin. *Exp Eye Res* 2008;87:376–384.
29. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, and Struhl JA: *Current Protocols in Molecular Biology*. 2006.
 30. Harris SA, Enger RJ, Riggs BL, and Spelsberg TC: Development and characterization of a conditionally immortalized human fetal osteoblastic cell line. *J Bone Miner Res* 1995;10:178–186.
 31. Veeman MT, Slusarski DC, Kaykas A, Louie SH, and Moon RT: Zebrafish *prickle*, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. *Curr Biol* 2003; 13:680–685.

Address correspondence to:

Dr. Rajiv Kumar
Nephrology Research, Department of Medicine
Mayo Clinic College of Medicine
Mayo Clinic
MS 1-120
200 First Street SW
Rochester, MN 55905

E-mail: rkumar@mayo.edu

Received: May 28, 2009

Accepted: June 29, 2009