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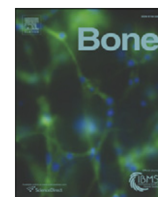

Original Full Length Article

Temporal changes in systemic and local expression of bone turnover markers during six months of sclerostin antibody administration to ovariectomized rats☆,☆☆

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A B S T R A C T

Sclerostin (Scl) is an osteocyte protein that decreases bone formation, and its inhibition by neutralizing antibodies (Scl-Ab) increases bone formation, mass and strength. We investigated the effects of Scl-Ab in mature ovariectomized (OVX) rats with a mechanistic focus on longer-term responses of osteoclasts, osteoblasts and osteocytes. Four-month-old Sprague–Dawley rats had OVX or sham surgery. Two months later, sham controls received sc vehicle while OVX rats received vehicle (OVX-Veh) or Scl-Ab (25 mg/kg) once weekly for 6 or 26 weeks followed by necropsy ($n = 12$/group). Terminal blood was collected for biochemistry, non-adherent marrow cells were harvested from femurs for ex vivo osteoclast formation assays, and vertebrae and tibiae were collected for dynamic histomorphometry and mRNA analyses. Scl-Ab treatment led to progressively thicker but fewer trabeculae in the vertebra, leading to increased trabecular bone volume and reduced trabecular surfaces. Scl-Ab also increased cortical bone volume in the tibia, via early periosteal expansion and progressive endocortical contraction. Scl-Ab significantly reduced parameters of bone resorption at week 6 relative to OVX-Veh controls, including reduced serum TRACP-5b, reduced capacity of marrow cells to form osteoclasts ex vivo, and >80% reductions in vertebral trabecular and tibial endocortical eroded surfaces. At week 26, serum TRACP-5b and ex vivo osteoclast formation were no longer reduced in the Scl-Ab group, but eroded surfaces remained >80% lower than in OVX-Veh controls without evidence for altered skeletal mRNA expression of *opg* or *rankl*. Scl-Ab significantly increased parameters of bone formation at week 6 relative to OVX-Veh controls, including increases in serum P1NP and osteocalcin, and increased trabecular, endocortical and periosteal bone formation rates (BFRs). At week 26, surface-referent trabecular BFR remained significantly increased in the Scl-Ab group versus OVX-Veh controls, but after adjusting for a reduced extent of trabecular surfaces, overall (referent-independent) trabecular BFR was no longer significantly elevated. Similarly, serum P1NP and osteocalcin were no longer significantly increased in the Scl-Ab group at week 26. Tibial endocortical and periosteal BFR were increased at week 6 in the Scl-Ab group versus OVX-Veh controls, while at week 26 only endocortical BFR remained increased. The Scl-Ab group exhibited significant increments in skeletal mRNA expression of several osteocyte genes, with *sost* showing the greatest induction in both the tibia and vertebra. We propose that Scl-Ab administration, and/or the gains in bone volume that result, may have increased osteocytic expression of Scl as a possible means of regulating gains in bone mass.

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Introduction

Sclerostin (Scl) is an endogenous inhibitor of osteoblasts that reduces bone formation and accrual of bone mass [1–3]. Therapeutic antibodies that inhibit Scl (Scl-Ab) can thus increase bone formation, and a variety of preclinical studies show that this effect leads to increased bone volume and bone strength (reviewed in [4]). Scl-Ab administration can also reduce bone resorption parameters, which may also contribute to improved bone mass and strength in animals [5,6]. Clinical studies in postmenopausal women showed that a single injection of Scl-Ab led to dose-dependent increases in bone formation markers, an acute reduction in the resorption marker serum CTx,

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and increased bone mineral density (BMD) [7,8]. Phase 3 clinical trials are currently underway to examine effects of Scl-Ab on bone resorption, bone formation, BMD and fracture risk in osteoporosis patients.

Animal studies demonstrate that the initial responses to Scl-Ab administration include substantial increases in the rates of bone formation on trabecular, endocortical and periosteal surfaces [5,6,9–12]. Studies examining the effects of longer-term Scl-Ab administration are beginning to emerge, suggesting that the stimulatory effects of Scl-Ab on osteoblasts may attenuate over time. Studies in postmenopausal women indicated that serum levels of the bone formation marker serum P1NP reached maximal increases within the first month of Scl-Ab administration, and gradually declined thereafter despite continued Scl-Ab dosing [8,13]. It is currently unclear whether tissue-level bone formation declines with similar kinetics in humans, and whether such a decline would occur uniformly throughout the skeleton. Data from animals treated with Scl-Ab indicate that histological parameters of bone formation can show more sustained stimulation relative to that of systemic biochemical markers of bone formation. For example, in an aged ovariectomized (OVX) rat study, the osteoblast marker serum osteocalcin increased in the early phase of Scl-Ab administration and returned to the levels of vehicle-treated controls thereafter, even though bone formation rates (BFRs) were significantly stimulated on trabecular, endocortical and periosteal surfaces [14]. Similarly, a study in cynomolgus monkeys demonstrated increased serum osteocalcin after 2 months of Scl-Ab administration, and this augmentation dissipated by month 6 of continued treatment despite histological evidence at the study's end point of increased BFR on endocortical (but not trabecular) surfaces [15]. Neither of these preclinical reports included bone histomorphometry analyses both early and late in the course of Scl-Ab treatment. The current study conducted in OVX rats provides novel longitudinal histomorphometry data indicating that the stimulation of tissue-level bone formation with Scl-Ab does indeed attenuate over time, in a compartment-specific manner. As cortical and trabecular bone volume increased via Scl-Ab administration, the extents of trabecular, endocortical and periosteal surfaces also changed in ways that reduced fidelity between surface-referent BFR values and systemic bone formation markers. To explore this influence, we also calculated BFRs using unbiased referent-independent approaches, the results of which seemed to better reflect the overall formation activities occurring within the cortical and trabecular compartments, and better reconcile with systemic markers of bone formation.

Mechanisms underlying attenuated osteoblast stimulation with longer-term Scl-Ab administration are currently unclear, and could have cellular, molecular and/or biomechanical bases. Osteocytes are the main cellular source of Scl, and Scl binds to LRP5/6 receptors on osteoblasts to inhibit their bone-forming activity in a manner that decreases the accrual of bone volume [1,4,16,17]. As Scl-Ab administration increases the volume of bone matrix, an absolute increase in the number of matrix-resident osteocytes will occur within the skeleton, which could increase the amount of Scl available to mitigate further increases in bone mass. Consistent with this possibility, recent studies indicated higher serum Scl levels in subjects with greater bone size and mass [18]. Additionally, or alternatively, if osteocytes recognize treatment-related increases in bone mass and strength via the attendant reduction in matrix strain [19], they may upregulate Scl expression as a means of controlling bone mass gains. This notion is supported by evidence that skeletal unloading, a state of reduced skeletal strain, led to increased osteocyte mRNA expression of the Scl gene *sost* [20].

In the interest of better understanding these phenomena, we treated mature OVX rats with Scl-Ab for 6 or 26 weeks and examined parameters of bone formation, bone resorption, bone volume and osteocyte gene expression. Ex vivo analyses of the osteoclast-forming potential of non-adherent bone marrow cells harvested from these animals provided new insights into possible mechanisms by which Scl-Ab reduces bone resorption.

Materials and methods

This study was conducted in accordance with federal animal care guidelines. The protocol and procedures were approved by Amgen's Institutional Animal Care and Use Committee.

Study design

Virgin female Sprague–Dawley rats were obtained from Harlan Laboratories (Indianapolis, IN, USA) and were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals*, 8th Edition [21]. All research protocols were approved by the Institutional Animal Care and Use Committee. Animals were pair-housed at Amgen's AAALAC-accredited facility in filter-top cages on corn cob bedding, with ad libitum access to pelleted feed (Harlan/Teklad 22/5 Rodent Diet, Madison, WI, USA) and reverse-osmosis purified water via an automatic watering system. Animals were maintained on a 12:12 hour light:dark cycle, and had access to enrichment opportunities. All animals were determined specific pathogen free. Rats were either sham-operated (Sham) or OVX at 4 months of age. At 6 months of age, the osteopenic OVX rats were assigned to treatment groups based on balanced body weights and areal BMD (determined in vivo by dual-energy X-ray absorptiometry [DXA]; Hologic QDR 4500a; Bedford, MA, USA). OVX rats were then treated with either vehicle or Scl-Ab (Scl-AbVI, 25 mg/kg, sc, 1×/week) for either 6 or 26 weeks ($n = 12$ per group), followed by necropsy. Sham rats were treated with vehicle ($n = 12$). Rats were injected sc with calcein (20 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) at 13 and 3 days before necropsy.

Bone histomorphometry

Undecalcified parasagittal 4-μm-thick sections of the third lumbar vertebral body and 6-μm-thick transverse sections of the right tibia at the tibiofibular junction were prepared as described previously [5]. Vertebral body sections were either stained with modified Goldner's Trichrome for analysis of static parameters or left unstained for collection of fluorochrome-based data. Tibial shaft sections were left unstained for collection of both fluorochrome-based and static parameter data. Histomorphometric analyses were made using Osteomeasure bone analysis software (Osteometrics, Inc.; Decatur, GA, USA). The region of interest (ROI) for the tibia included the entire periosteal and endocortical perimeters. The ROI for lumbar vertebral body trabecular bone included all trabecular bone within the parasagittal section that was located more than 0.5 mm from the endocortical surface. Static and dynamic parameters were calculated and expressed with traditional surface or volume referents in accordance with standard methods [22,23]. It is recognized that commonly used referents such as bone volume (BV) and bone surface (BS) can have limitations in terms of examining in detail the relationships between histological and biochemical indices of bone remodeling at the whole-body level [22,23]. In the current study, Scl-Ab treatment led to markedly increased bone volume and significant changes in the extents of trabecular, periosteal and endocortical surfaces. We thus explored whether non-conventional parameters of bone formation and resorption exhibited greater fidelity with systemic biochemical marker responses to Scl-Ab. Non-conventional parameters without a stated referent (Tb.BFR, Tb.ES, Ec.BFR, Ps.BFR, Ct.BFR, Ec.ES) represent absolute values collected for the entire ROI, regardless of its size. Because the trabecular ROI was modestly impacted by endocortical bone loss and gain in the OVX and Scl-Ab groups, respectively, the actual ROI area (i.e., total tissue area [T.Ar]) was applied as another alternative referent for trabecular parameters.

Regulators and markers of bone turnover in serum

Prior to necropsy, and 48 h after the last dose, animals were fasted overnight. Rats were gently warmed in their cages for 10–15 min

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