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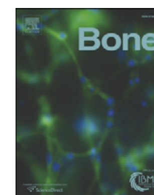

Original Full Length Article

Effect of anti-sclerostin therapy and osteogenesis imperfecta on tissue-level properties in growing and adult mice while controlling for tissue age

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A B S T R A C T

Bone composition and biomechanics at the tissue-level are important contributors to whole bone strength. Sclerostin antibody (Scl-Ab) is a candidate anabolic therapy for the treatment of osteoporosis that increases bone formation, bone mass, and bone strength in animal studies, but its effect on bone quality at the tissue-level has received little attention. Pre-clinical studies of Scl-Ab have recently expanded to include diseases with altered collagen and material properties such as osteogenesis imperfecta (OI). The purpose of this study was to investigate the role of Scl-Ab on bone quality by determining bone material composition and tissue-level mechanical properties in normal wild type (WT) tissue, as well as mice with a typical OI Gly ➔ Cys mutation (Brtl/+) in type I collagen. Rapidly growing (3-week-old) and adult (6-month-old) WT and Brtl/+ mice were treated for 5 weeks with Scl-Ab. Fluorescent guided tissue-level bone composition analysis (Raman spectroscopy) and biomechanical testing (nanoindentation) were performed at multiple tissue ages. Scl-Ab increased mineral to matrix in adult WT and Brtl/+ at tissue ages of 2–4 wks. However, no treatment related changes were observed in mineral to matrix levels at mid-cortex, and elastic modulus was not altered by Scl-Ab at any tissue age. Increased mineral-to-matrix was phenotypically observed in adult Brtl/+ OI mice (at tissue ages > 3 wks) and rapidly growing Brtl/+ (at tissue ages > 4 wks) mice compared to WT. At identical tissue ages defined by fluorescent labels, adult mice had generally lower mineral to matrix ratios and a greater elastic modulus than rapidly growing mice, demonstrating that bone matrix quality can be influenced by animal age and tissue age alike. In summary, these data suggest that Scl-Ab alters the matrix chemistry of newly formed bone while not affecting the elastic modulus, induces similar changes between Brtl/+ and WT mice, and provides new insight into the interaction between tissue age and animal age on bone quality.

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1. Introduction

Bone fragility is regulated across multiple hierarchical scales. In addition to bone mass, changes in material-level composition and biomechanics are critical determinants of whole bone strength [1]. Sclerostin antibody (Scl-Ab) is a novel anabolic candidate therapy for the treatment of osteoporosis. Experiments in a variety of animal models, as well as Phase I and Phase II clinical trials, demonstrate that Scl-Ab stimulates bone formation and increases bone mass [2–9]. However, the effect of Scl-Ab on tissue-level changes to bone composition and mechanics has received limited attention, with little, or no, change in mineralization (backscattered scanning electron microscopy) and material composition (Fourier transform infrared spectroscopy) reported in rats, primates, or OI mice treated with Scl-Ab [10,11]. While SOST-KO mice and patients with sclerosteosis represent extreme examples of lifelong absence of sclerostin unlikely to be replicated by periodic Scl-Ab dosing, data from these mice and human patients support the potential for sclerostin-related alterations in bone material composition [12].

Osteogenesis imperfecta (OI), also known as "brittle bone disease," is a genetic collagen-related disorder which causes fragile bones and frequent fractures in children [13]. The increased fracture risk of OI is often a product of reduced bone mass and altered material composition. At the matrix level, despite decreases in overall bone mass, OI patients and animal models typically reflect an increased level of tissue mineralization [14–17] which is believed to be associated with tissue brittleness

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characteristic of the disease. To study the potential anabolic efficacy of Scl-Ab treatment in OI, pre-clinical studies in the Brtl/+ [18–20], Amish [21], and JRT [11] mouse models of OI have been performed. The Brtl/+ model of Type IV OI is heterozygous for a typical Gly ➔ Cys substitution on col1a1 (G349C) and recapitulates many features of the OI phenotype including reduced bone mass, reduced bone strength, and increased bone resorption relative to bone formation [22–24]. We previously showed that Scl-Ab increased bone formation and bone mass in Brtl/+, and mechanical four-point bending revealed that Scl-Ab significantly reduced bone brittleness in young WT, and adult Brtl/+ and WT mice [19,20]. These findings suggested potential alterations at the material level that have yet to be fully explored.

The purpose of this study was to determine the effect of Scl-Ab on tissue-level mechanical properties and material composition in normal WT tissue, as well as Brtl/+ OI mice using nanoindentation and Raman spectroscopy. As bone material properties and OI fracture risk change with age, both rapidly growing (3-week-old) and adult (6-month-old) ages of WT and Brtl/+ were studied. Fluorescent-guided bone material analysis was used to rigorously control for tissue age and to determine any differences in mineralization dynamics. Analyzing samples at the same tissue ages in both rapidly growing and adult animals enabled insight into the direct interaction between tissue age and animal age on tissue level bone properties. This study adds new data describing Scl-Ab effects on tissue level material composition and biomechanics, investigates Scl-Ab effects on OI tissue, and highlights the interaction between animal age and tissue age during bone formation.

2. Materials and methods

2.1. Animals

Wildtype (WT) and Brtl/+ [22] mice are maintained on a mixed background of Sv129/CD-1/C57BL/6S, and all Brtl/+ animals were the product of breeding male heterozygous Brtl/+ with female WT. 3 week and 6 month old male WT and Brtl/+ mice were randomly assigned to Scl-Ab (Scl-Ab VI, Amgen, Thousand Oaks, CA) treatment or vehicle injection (PBS). Sclerostin antibody was injected subcutaneously at 25 mg/kg, two times per week, for five weeks. Calcein (30 mg/kg, i.p. injection) was injected at the start of experiment (5 weeks before euthanasia), after 2 weeks of treatment (3 weeks before euthanasia), and after 4 weeks of treatment (1 week before euthanasia). A final alizarin label (30 mg/kg, i.p. injection) was given 1 day before euthanasia. A summary of the experimental design is shown in Fig. 1A. The multiple fluorescent labels were used to visualize the growth pattern during the entire course of therapy. Body weights were recorded with each injection. Right femurs were harvested and stored at −20 °C in lactated Ringers solution (LRS) soaked gauze until testing or further specimen preparation. All protocols and procedures involving animals were approved by the University of Michigan's Committee on Use and Care of Animals.

2.2. Specimen preparation

Two distinct cohorts of animals were used for this study. Nanoindentation was performed on the same animals previously described [19,20]. These samples underwent 2–4 freeze cycles in the course of harvesting and specimen preparation, but this was not rigorously controlled for. Nanoindentation group sizes are n = 7–9/group, before accounting for testing errors. All Raman spectroscopy tests were performed on a second cohort of animals (both 3-week-old and 6-month-old) that were treated as described above except that femora had only one freeze/thaw cycle prior to imaging to avoid confounding the results [25]. All Raman spectroscopy data was n = 5–7/group.

Right femora were thawed, encased in a quick setting (~30 min) epoxy (Kold Mount, Vernon-Benshoff, Albany, NY) without dehydration, and cut transversely at the mid-diaphysis with a low-speed saw (IsoMet, Beuhler, Lake Bluff, IL). The distal section of tissue was polished using progressive grades of silicon carbide abrasive paper (1200, 2400, and 4000 grit) under water irrigation for 2 min at each grade. Each specimen was polished to a plane just distal to the third trochanter, identical to that performed by dynamic histomorphometry, and similar to a plane included in our cortical microCT and mechanical 4 pt. testing data previously reported [19,20].

To further decrease surface roughness for nanoindentation, the encased specimens were polished on a felt pad for 5 min with a 1/4 μm diamond suspension (Struers Inc., Cleveland, OH). Specimens were then ultrasonically cleansed in a water bath for 10 min to remove surface debris, and glued to a glass slide specimen plate for nanoindentation testing in a custom-made hydration chamber.

2.3. Nanoindentation

A custom TI 950 TriboIndenter (Hysitron, Minneapolis, MN) instrumented with a fluorescent light-source and FITC filter allowed for simultaneous visualization of calcein labeling in specimens and accurate positioning of indents to locations matched for tissue age and treatment status with 0.5 μm spatial resolution. Five (rapidly growing) or six (adult) regions of interest were mechanically tested in the posterior aspect of the femoral cross section (Fig. 1B): the mid-cortex (defined as midway between the first calcein label on the periosteal and endosteal surfaces, if any), 15 μm endosteal to ("inside") the first 5 wk old label, along the first calcein label on 5 wk old bone, along the second calcein label on 3 wk old bone, along the third calcein label on 1 wk old bone, and along the final alizarin label on 1 day old bone. Due to technical constraints on the nanoindentation fluorescent imaging hardware, the outer alizarin label positions were identified by images taken on a separate microscope (Zeiss Axiovert) and measuring the distance from the 1 wk old calcein label. The mid-cortex and 15 μm endosteal to the 5 wk label positions were nearly identical locations in rapidly growing 3 week animals. As such, nanoindentation values were only collected at one location and characterized as "mid-cortex" in all analysis. As Veh treated 6 mo old animals grow much slower than 3 wk old animals, it was not always possible to delineate the outer 1 wk and 1 day old labels in adult 6 mo mice as they appeared in the same location. Moreover, nanoindentation requires spacing between indents to not indent on the tissue deformed by a previous indent. Therefore, in these instances, data was only collected in one location, and it was treated as a tissue age of 1 wk. As a result, there is reduced statistical power with n = 5 for Adult WT Veh at a tissue age of 1 day and an n = 3 for Adult Brtl/+ Veh at a tissue age of 1 day, instead of the n = 7–9 for all other nanoindentation outcomes.

Indentation consisted of a 10 μN pre-load, with a diamond Berkovich indenter tip into samples, followed by loading the sample at 300 μN/s, hold at a maximal load of 3000 μN for 10 s, and unloading at 300 μN/s. All indents were performed on samples fully hydrated with saline using a custom hydration chamber. The indentation modulus E was calculated from the load–displacement curves using the standard Oliver–Pharr method [26]. Quartz (E = 69.6 GPa) was used to define the tip-area function, and was also used throughout testing to ensure consistent calibration of the machine. Eight indents, 10 μm apart, were made along each fluorescent label and region of interest, with values averaged for each site and mouse.

2.4. Raman spectroscopy

Raman measurements were performed with a custom microscope outfitted for Raman spectroscopy as previously described [27]. Briefly, Raman spectra were collected by directing a 785-nm stabilized diode laser (Invictus, Kaiser Optical Systems, Inc., Ann Arbor, MI) through a Nikon E600 microscope (Nikon Instruments Inc., Melville, NY) with a 40×/0.90 NA objective (S Fluor, Nikon Instruments, Inc., Melville, NY) operated in epi-illumination/collection mode. Collected light from the

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