Bone Reports 8 (2018) 90–94

Contents lists available at ScienceDirect

Bone Reports

journal homepage: www.elsevier.com/locate/bonr

Decreased osteoprogenitor proliferation precedes attenuation of cancellous bone formation in ovariectomized rats treated with sclerostin antibody

Rogely Waite Boyce[a,*], Danielle Brown[b], Melanie Felx[c], Nacera Mellal[c], Kathrin Locher[a], Ian Pyrah[a,1], Michael S. Ominsky[d,2], Scott Taylor[a]

[a] Department of Comparative Biology and Safety Sciences, Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 91320, United States
[b] Charles River Laboratories, 4025 Stirrup Creek Drive, Suite 150, Durham, NC 27703, United States
[c] Charles River Laboratories, 22022 Transcanadienne, Senneville, QC H9X 3R3, Canada
[d] Department of CardioMetabolic Disorders, Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 91320, United States

ARTICLE INFO

Keywords:
Osteoporosis
Anabolics
Cell signaling
Osteoprogenitors
Wnt signaling
Bone

ABSTRACT

Sclerostin antibody (Scl-Ab) stimulates bone formation, which with long-term treatment, attenuates over time. The cellular and molecular mechanisms responsible for the attenuation of bone formation are not well understood, but in aged ovariectomized (OVX) rats, the reduction in vertebral cancellous bone formation is preceded by a reduction in osteoprogenitor (OP) number and significant induction of signaling pathways known to suppress mitogenesis and cell cycle progression in the osteocyte (OCy) (Taylor et al., 2016). To determine if the reduction in OP number is associated with a decrease in proliferation, aged OVX rats were administered vehicle or Scl-Ab for 9 or 29 days and implanted with continuous-delivery 5-bromo-2′-deoxyuridine (BrdU) mini-osmotic pumps 5 days prior to necropsy. The total number of BrdU-labeled osteoblasts (OB) was quantified in vertebral cancellous bone to indirectly assess the effects of Scl-Ab treatment on OP proliferation at the time of activation of modeling-based bone formation at day 9 and at the time of maximal mineralizing surface, initial decrease in OP number, and transcriptional changes in the OCy at day 29. Compared with vehicle, Scl-Ab resulted in an increase in the total number of BrdU-positive OB (+260%) at day 9 that decreased with continued treatment (+50%) at day 29. These differences in proliferation occurred at time points when the increase in total OB number was significant and similar in magnitude. These findings suggest that reduced OP proliferation contributes to the decrease in OP numbers, an effect that would limit the OB pool and contribute to the attenuation of bone formation that occurs with long-term Scl-Ab treatment.

1. Introduction

Sclerostin antibody (Scl-Ab) stimulates bone formation, largely by increasing modeling-based bone formation on cancellous and cortical bone surfaces (Boyce et al., 2017; Ominsky et al., 2017a; Ominsky et al., 2014). The increase in bone formation is transient, with bone formation attenuating with long-term treatment. The progressive decline in bone formation in response to Scl-Ab displays envelope-specific behavior. In rats and monkeys administered Scl-Ab, bone formation attenuates first on the cancellous bone surfaces, followed by a more delayed attenuation on the cortical surfaces (Chouinard et al., 2016; Li et al., 2014; Ominsky et al., 2017b).

The cellular and molecular basis of the progressive decline in bone formation with long-term Scl-Ab treatment is not completely understood. In rats, long-term Scl-Ab treatment and the progressive decrease in cancellous bone formation is associated with decreases in total number of osteoprogenitors (OP) in the vertebrae (Ominsky et al., 2015; Taylor et al., 2016). In aged ovariectomized (OVX) rats treated

Abbreviations: ANOVA, analysis of variance; BrdU, 5-bromo-2′-deoxyuridine; CDKN1A, cyclin-dependent kinase inhibitor 1A; CDKN2A, CDKN inhibitor 2A; CE, coefficient of error; CV, coefficient of variation; D, day; E2F1, E2F transcription factor 1; FOXM1, Forkhead box protein M1; MS/BS, mineralizing surface per bone surface; MYC, v-myc avian myelocytomatosis viral oncogene homolog; MYCN, MYC neuroblastoma-derived homolog; OB, osteoblast(s); Ob.N, OB number; OCy, osteocyte(s); OP, osteoprogenitor(s); OVX, ovariectomized; PROBE, precision range of an optimally balanced estimator; RB1, retinoblastoma protein 1; RUNX2, Runt-related transcription factor 2; Scl-Ab, sclerostin antibody; Scl-AbVI, 50 mg/kg of a Scl-Ab; SURS, systematic uniform random sampling; TP53, tumor protein p53; VEH, vehicle

* Corresponding author at: Department of Comparative Biology and Safety Sciences, Amgen Inc., One Amgen Center Drive, MS 29-2-A, Thousand Oaks, CA 91320, United States.
E-mail addresses: rboyce@amgen.com (R.W. Boyce), Danielle.Brown@crl.com (D. Brown), Melanie.Felx@crl.com (M. Felx), Nacera.Mellal@crl.com (N. Mellal), kathrinl@amgen.com (K. Locher), ipyrah@seagen.com (I. Pyrah), ominsky@umich.edu (M.S. Ominsky), taylorsc@amgen.com (S. Taylor).
[1] Current address: Seattle Genetics, 21823-30th Drive S.E., Bothell, WA 98021, United States.
[2] Current address: Radius Health, Inc., 950 Winter St, Waltham, MA 02451, United States.

https://doi.org/10.1016/j.bonr.2018.03.001
Received 29 August 2017; Received in revised form 15 February 2018; Accepted 2 March 2018
Available online 03 March 2018

with Scl-Ab for up to 183 days, reduction in OP numbers preceded the reduction in osteoblast (OB) numbers and attenuation of bone formation in vertebral cancellous bone. The initial reduction in OP numbers occurred at the time of maximal bone formation rate and was coincident with significant induction of signaling pathways in the osteocyte (OCy) known to regulate Wnt signaling and suppress mitogenesis and cell cycle progression (Taylor et al., 2016). Induction of these pathways was unique to the OCy and was not observed in the OB or lining cell, other terminally differentiated, nonproliferating cells of the OB lineage (Taylor et al., 2016).

It is unknown if the reduction in OP numbers in cancellous bone marrow is temporally associated with reduced OP proliferation, an effect that would reduce the OB pool and potentially contribute to the progressive attenuation of bone formation with Scl-Ab. To assess the effects of Scl-Ab on OP proliferation, we used 5-bromo-2′-deoxyuridine (BrdU) labeling to quantify total number of BrdU-labeled OB in lumbar vertebral cancellous bone as an indirect assessment of OP proliferation in response to Scl-Ab. Analyses were conducted early in the course of treatment at the time of activation of modeling-based bone formation and later at the time corresponding to the initial decrease in OP numbers, maximal mineralizing surface, and transcriptional changes in the OCy.

2. Materials and methods

2.1. Study design

Six-month-old female Sprague-Dawley rats (SD®IGS; Charles River Laboratories, Raleigh, NC, USA) were OVX and left untreated for 8 weeks. Rats were assigned to four treatment groups in a manner to achieve body weight balance across the treatment groups. Rats were administered vehicle (VEH) or 50 mg/kg of Scl-Ab (Scl-AbVI) by weekly subcutaneous injection. Scl-AbVI was engineered to be less immunogenic in rats (rat fragment crystallizable construct) by modifying the murine parent antibody to be more similar to that found in rats.

Two groups received a single dose of VEH (n = 12) or Scl-Ab (n = 11) and were euthanized on day 9, and two groups received four doses of VEH (n = 11) or Scl-Ab (n = 12) and were euthanized on day 29.

Five days prior to the scheduled necropsies, Alzet® pumps (Model 2ML1 with an infusion rate of 10 µL/h; Cupertino, CA, USA), each containing 2 mL of BrdU at 50 mg/mL in Dulbecco's phosphate buffered saline and 15% dimethyl sulfoxide, were implanted subcutaneously in the interscapular area of all rats. For a rat weighing 500 g, the estimated daily dose of BrdU was 24 mg/kg. Pumps were weighed before and after filling and after removal at necropsy to verify the delivery. Two animals were excluded from subsequent analyses (one day 29 VEH and one day 9 Scl-Ab) due to evidence of pump failure. For euthanasia, animals were anesthetized with isoflurane/oxygen and then exsanguinated. The 5-day labeling period was chosen because it approximates the estimated transit time from OP to mature OB (approximately 7 days) based on lineage-tracing studies in Sox9creER^{T2}, Rosa26-tdTomato, osteocalcin–green fluorescent protein triple-transgenic mice (Kronenberg and Balani, 2015).

The in-life phase of this study was conducted at Charles River Laboratories (Montreal ULC, Canada). Animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals, 8th Edition (Committee for the Update of the Guide of the Care and Use of Laboratory Animals: National Research Council (US), 2011). All research protocols were approved by the Institutional Animal Care and Use Committee. Animals were group-housed (two per cage) at an Association for Assessment and Accreditation of Laboratory Animal Care, internationally accredited facility, in plastic cages and then transferred to sterile, ventilated, micro-isolator housing with corncob bedding following implantation of Alzet® pumps. Animals had ad libitum access to pelleted feed (Certified Rodent Chow No. 5CR4 [14% protein]; PMI Nutrition International, St. Louis, MO, USA) and water (reverse osmosis purified) using an automatic watering system. Animals were maintained on a 12:12 hour light:dark cycle in rooms with controlled temperature (19°–25 °C) and humidity (30%–70%) and access to enrichment opportunities (hiding tube and a chewing object). Further details on animal care and surgical procedures are provided in Supplementary materials.

2.2. Estimation of total number of BrdU-labeled OB

At necropsy, lumbar vertebrae L1-L2 were isolated and cleaned of the soft tissue; vertebral body segment was isolated, fixed in 10% neutral buffered formalin, and transferred to 70% ethanol. A segment of the duodenum was collected and processed routinely in paraffin to use as a positive control to verify BrdU incorporation. The vertebral segments were decalcified in modified Kristensen's solution, processed in filtered paraffin, and embedded longitudinally in paraffin blocks.

At days 9 and 29 in this study, the total number of BrdU-labeled OB was quantified in vertebral cancellous bone. For quantification, the stereological estimator, the physical fractionator (Gundersen, 1986), was used. Using a random starting point, serial pairs of 3-µm thick sections (physical disectors) were collected, spaced 240 µm apart, by systematic uniform random sampling (SURS), yielding approximately 8–10 disector pairs per animal. Because stereological analyses required exhaustive sectioning of the sample and generation of thin serial section pairs, vertebrae were decalcified to facilitate sectioning and avoid section artifacts, and thus, osteoid surfaces or fluorochrome labeling could not be used to identify active forming surfaces. Therefore, to identify BrdU-labeled OB active in matrix synthesis, a double immunohistochemical staining was performed to colocalize BrdU-positive nuclei in osteonectin-positive OB lining the cancellous bone surface (Fig. 1).

For immunostaining, disector pairs were retrieved offline in Diva Decloaker (Biocare Medical, Concord, CA, USA) for 18 h, rinsed, incubated with 3% H_2O_2 for 10 min, rinsed with buffer, and then transferred to a Ventana Discovery XT (Ventana Medical Systems, Inc., Tucson, AZ, USA). The BrdU immunoreactivity was detected by incubating with an anti-BrdU antibody for 1 h (ab1893; Abcam, Cambridge, MA, USA), followed by biotin-conjugated rabbit antisheep secondary antibody for 32 min (item number 313-065-003; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and then horseradish peroxidase–labeled streptavidin for 16 min followed by

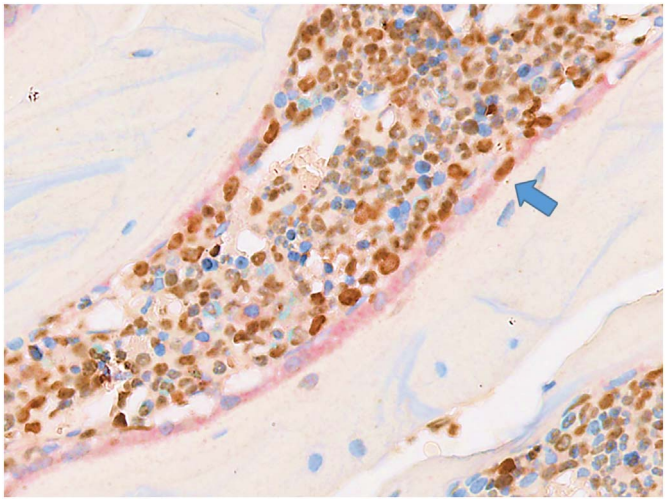

Fig. 1. Double immunohistochemical staining for osteonectin and BrdU to identify BrdU-positive osteoblasts active in matrix synthesis. Osteonectin-positive osteoblasts stained with Fast Red line the cancellous bone surfaces in a Scl-Ab–treated rat, with a BrdU-positive osteoblast (blue arrow, original magnification 400×). BrdU = 5-bromo-2′-deoxyuridine; Scl-Ab = sclerostin antibody.