Bone 107 (2018) 93–103

Contents lists available at ScienceDirect

Bone

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

Full Length Article

Dampening of the bone formation response following repeat dosing with sclerostin antibody in mice is associated with up-regulation of Wnt antagonists

Gill Holdsworth [a,*,1], Kevin Greenslade [a,1], Joby Jose [a], Zofia Stencel [a], Hishani Kirby [a,b], Adrian Moore [a], Hua Zhu Ke [a], Martyn K. Robinson [a]

[a] UCB Pharma, Slough, UK
[b] Hishani Kirby Associates Ltd, Reading, UK

ARTICLE INFO

Article history:
Received 20 June 2017
Revised 3 October 2017
Accepted 5 November 2017
Available online 09 November 2017

Keywords:
Sclerostin antibody
Bone formation
Wnt antagonists
Osteoporosis

ABSTRACT

Administration of antibodies to sclerostin (Scl-Ab) has been shown to increase bone mass, bone mineral density (BMD) and bone strength by increasing bone formation and decreasing bone resorption in both animal studies and human clinical trials. In these studies, the magnitude and rate of increase in bone formation markers is attenuated upon repeat dosing with Scl-Ab despite a continuous and progressive increase in BMD.

Here, we investigated whether the attenuation in the bone formation response following repeated administration of Scl-Ab was associated with increased expression of secreted antagonists of Wnt signalling and determined how the circulating marker of bone formation, P1NP, responded to single, or multiple doses, of Scl-Ab four days post-dosing.

Female Balb/c mice were treated with Scl-Ab and we demonstrated that the large increase in serum P1NP observed following the first dose was reduced following administration of multiple doses of Scl-Ab. This dampening of the P1NP response was not due to a change in the kinetics of the bone formation marker response, or differences in exposure to the drug.

The abundance of transcripts encoding several secreted Wnt antagonists was determined in femurs collected from mice following one or six doses of Scl-Ab, or vehicle treatment. Compared with vehicle controls, expression of *SOST*, *SOST-DC1*, *DKK1*, *DKK2*, *SFRP1*, *SFRP2*, *FRZB*, *SFRP4* and *WIF1* transcripts was significantly increased (approximately 1.5–4.2 fold) following a single dose of Scl-Ab. With the exception of *SFRP1*, these changes were maintained or further increased following six doses of Scl-Ab and the abundance of *SFRP5* was also increased. Up-regulation of these Wnt antagonists may exert a negative feedback to increased Wnt signalling induced by repeated administration of Scl-Ab and could contribute to self-regulation of the bone formation response over time.

After an antibody-free period of four weeks or more, the P1NP response was comparable to the naïve response, and a second phase of treatment with Scl-Ab following an antibody-free period elicited additional gains in BMD. Together, these data demonstrate that the rapid dampening of the bone formation response in the immediate post-dose period which occurs after repeat dosing of Scl-Ab is associated with increased expression of Wnt antagonists, and a treatment-free period can restore the full bone formation response to Scl-Ab.

1. Introduction

Causative loss-of-function mutations in the *SOST* gene have been identified in the rare inherited high bone mass condition called sclerosteosis [1,2]; a condition found primarily in an Afrikaner population in South Africa. The product of the *SOST* gene – sclerostin – is a secreted glycoprotein primarily produced by bone-dwelling osteocytes, which binds to the low density lipoprotein receptor-related protein 4/5/6 (LRP4/5/6) Wnt co-receptors to act as a secreted antagonist of canonical Wnt signalling [3–5]. Consistent with the known effect of

Abbreviations: BMD, bone mineral density; *Col1a1*, collagen, type I, alpha 1; Dkk, Dickkopf; DXA, dual-energy X-ray absorptiometry; *Eif3f*, eukaryotic translation initiation factor 3, subunit F; FRZB, Frizzled related protein; LRP, low density lipoprotein receptor-related protein; P1NP, procollagen type 1 *N*-terminal-propeptide; *Psmd4*, proteasome (prosome, macropain) 26S subunit, non-ATPase, 4; *Rpl38*, ribosomal protein L38; Scl-Ab, sclerostin antibody; SFRP, secreted frizzled-related protein; SOST-DC1, sclerostin domain-containing protein 1; WIF, Wnt inhibitory factor 1.

* Corresponding author.
E-mail address: Gill.Holdsworth@ucb.com (G. Holdsworth).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.bone.2017.11.003
8756-3282/

sclerostin as an inhibitor of bone formation, the life-long absence of sclerostin in individuals with sclerosteosis results in a bone overgrowth phenotype with some affected individuals showing bone mineral density (BMD) *Z*-scores in excess of +14 [6]. High bone mass was also seen in mice in which recombinant DNA techniques had been used to inactivate the gene encoding sclerostin [7] whilst transgenic mice over-expressing the SOST gene display decreased bone mass and strength [8].

Administration of antibodies to sclerostin (Scl-Ab) has been shown to increase bone formation and decrease bone resorption, leading to a rapid increase in bone mass, BMD and bone strength in a variety of pre-clinical animal studies (reviewed by Ke et al. [9] and Ominsky et al. [10]). In human clinical trials, sclerostin antibodies have been shown to rapidly increase bone mineral density and decrease the incidence of osteoporotic fractures [11,12]. Treatment with Scl-Ab causes a rise in markers of bone formation but these generally return to near baseline after extended treatment [11–14]. The initial rate of bone formation following exposure to Scl-Ab is very high and histological studies in preclinical models have demonstrated that this rate declines or self-regulates after multiple antibody administrations [15]. It is likely that multiple mechanisms contribute to self-regulation of the bone formation response with long-term treatment of Scl-Ab.

Wnt signalling plays an important role in bone homeostasis, with the canonical arm of this pathway affecting the entire osteoblast lineage (reviewed by Baron and Kneissel [16]). Canonical Wnt signalling is known to be regulated by a wide variety of effector molecules [17] and transcriptional profiling studies have provided some insights into the potential for altered expression in components of this pathway, and pathways downstream of canonical Wnt signalling, to contribute to the self-regulation of the bone-forming response observed with chronic dosing of Scl-Ab. Recent pre-clinical studies conducted in rats have shown that treatment with Scl-Ab results in progressive up-regulation of two secreted antagonists of Wnt signalling: *SOST* and *Dickkopf1* (*Dkk1*) in osteoblast lineage cells [18–20] in intact tibia and vertebrae, as well as in vertebral trabecular bone.

It is not known whether induction of additional secreted inhibitors of Wnt signalling occurs following up-regulation of this signalling pathway in bone through administration of Scl-Ab, or whether attenuation of the bone-forming response to Scl-Ab is reversed by an antibody-free period. In this study, we treated mice with Scl-AbI [21] and examined the effect on BMD and showed that the bone formation marker, procollagen type 1 *N*-terminal-propeptide (P1NP), displayed pulsatile increases in the immediate post-dose period and the magnitude of this increase rapidly and progressively decreased following repeat dosing with the antibody. Retreatment following an antibody-free period was able to restore the full bone formation response and resulted in further gains in BMD. Most importantly, we investigated how single, or multiple doses of Scl-AbI affected skeletal expression of transcripts encoding secreted antagonists of Wnt signalling. Due to technical challenges associated with the analysis of trabecular bone in mice, we analysed changes which occurred in cortical bone. The data presented here will further our understanding around the mechanisms affecting the bone formation response following treatment with Scl-Ab.

2. Materials and methods

2.1. Study design

Female Balb/c mice were used in all experiments (supplied by Charles River, 8–10 weeks old at start of experiments). Mice were housed in cages in an environmentally controlled room (temperature 21 °C to 23 °C and relative humidity 38% to 50% on a 12-hour light/dark cycle according to UK Home Office regulations). Animals had access to RM1 food pellets (Lillico) and water ad libitum. Animals were acclimatised for a minimum of one week before use. All animal experiments were performed in agreement with UK guidelines (Animals (Scientific Procedures) Act 1986) and were approved by the UCB Pharma Animal Welfare and Ethical Review Body. Scl-AbI [21] was administered at 10 mg/kg s.c. weekly. Phosphate buffered saline (PBS) was administered s.c. to vehicle control groups whenever Scl-AbI was administered.

2.2. Measurement of serum bone formation marker

P1NP (Procollagen type 1) was measured using a competitive ELISA kit (AC-33F1) from ImmunoDiagnostic Systems according to manufacturer's recommendations. Peak P1NP levels were routinely measured 4 days after antibody dosing. Plasma levels of Scl-Ab were measured using an antigen-binding ELISA. Bone mineral density (BMD) was measured using LUNAR PIXImus DXA scanner.

2.3. Bone RNA isolation

Left and right femurs isolated from mice were cleaned of soft tissue before removal of the proximal and distal epiphyseal regions. The remaining diaphysis region was flushed with 2 ml cold PBS using a needle to remove bone marrow and the femur shaft was flash frozen in liquid nitrogen. Left and right femur shafts from each mouse were combined and total RNA was isolated from these pooled femurs via 2 × 3 min homogenisation in 1 ml Qiazol lysis reagent (Qiagen) using 5 mm stainless steel beads (Qiagen) and TissueLyser II (Qiagen). Following the addition of chloroform, samples were centrifuged (12,000 × *g* for 15 min at 4 °C) and the aqueous phase retained and RNA therein precipitated by the addition of propan-2-ol and centrifugation (12,000 × *g* for 10 min at 4 °C). Total RNA extracted in this way was further purified using RNeasy Plus Mini kit (Qiagen) as per the manufacturer's instructions.

2.4. Quantitative real-time RT-PCR (TaqMan)

A two-step TaqMan protocol was used. RNA was reverse transcribed using SuperScript VILO cDNA synthesis kit (Life Technologies) in accordance with the manufacturer's instructions. TaqMan PCR was performed in triplicate wells using TaqMan Gene Expression Master Mix and the TaqMan Gene Expression Assays (Life Technologies) shown in Table 1. 1 μl sample cDNA/well was used in a final volume of 10 μL per well. No RT and no template control wells were included in each experiment. The ΔΔCt method was used to normalise expression against the geometric mean of *Eif3f* and *Psmd4*, which were reported as being constitutively expressed housekeeping genes in a range of tissues by Kouadjo et al. [22]. Data from femur pools for each animal group were normalised relative to transcript abundances observed in the group receiving vehicle control.

Table 1
Table of probes used for quantitative real-time PCR experiments.

Gene symbol	Gene name	Assay ID
SOST	Sclerostin	Mm04208528_m1
SOST-DC1	Sclerostin domain containing protein 1	Mm00840254_m1
Dkk1	Dickkopf homolog 1	Mm00438422_m1
Dkk2	Dickkopf homolog 2	Mm01322146_m1
Dkk4	Dickkopf homolog 4	Mm00461141_m1
SFRP1	Secreted frizzled-related protein 1	Mm00489161_m1
SPRF2	Secreted frizzled-related protein 2	Mm01213947_m1
FRZB	Frizzled-related protein	Mm00441378_m1
SPRF4	Secreted frizzled-related protein 4	Mm00840104_m1
SFRP5	Secreted frizzled-related protein 5	Mm01194236_m1
WIF1	Wnt inhibitory factor 1	Mm00442355_m1
Col1a1	Collagen, type I, alpha 1	Mm00801666_g1
Eif3f	Eukaryotic translation initiation factor 3, subunit F	Mm01263490_m1
Psmd4	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 4	Mm00517953_m1
Rpl38	Ribosomal protein L38	Mm03015864_g1