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Anti-DKK1 antibody promotes bone fracture healing through activation of β-catenin signaling

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ABSTRACT

In this study we investigated if Wnt/β-catenin signaling in mesenchymal progenitor cells plays a role in bone fracture repair and if DKK1-Ab promotes fracture healing through activation of β-catenin signaling. Unilateral open transverse tibial fractures were created in CD1 mice and in *β-catenin*^{Prx1ER} conditional knockout (KO) and Cre-negative control mice (C57BL/6 background). Bone fracture callus tissues were collected and analyzed by radiography, micro-CT (μCT), histology, biomechanical testing and gene expression analysis. The results demonstrated that treatment with DKK1-Ab promoted bone callus formation and increased mechanical strength during the fracture healing process in CD1 mice. DKK1-Ab enhanced fracture repair by activation of endochondral ossification. The normal rate of bone repair was delayed when the *β-catenin* gene was conditionally deleted in mesenchymal progenitor cells during the early stages of fracture healing. DKK1-Ab appeared to act through β-catenin signaling to enhance bone repair since the beneficial effect of DKK1-Ab was abrogated in *β-catenin*^{Prx1ER} conditional KO mice. Further understanding of the signaling mechanism of DKK1-Ab in bone formation and bone regeneration may facilitate the clinical translation of this anabolic agent into therapeutic intervention.

Introduction

Skeletal fractures may occur as a consequence of trauma as well as fragility and represent a significant public health problem. There are over 6 million adults that suffer fractures in the United States annually [1–3]. These fractures can be associated with significant morbidity, costs, and health care utilization [4], especially when people with poor outcomes suffer with fractures.

Fracture healing is a specialized postnatal repair process that recapitulates aspects of embryonic skeletal development [5–9]. Wnt/β-catenin is one of the critical signaling pathways that regulate chondrogenesis, osteogenesis, and osteoclast formation. Clinical interest in this pathway was sparked by the discovery that osteoporosis pseudoglioma (OPPG), a disease characterized by low bone mass and recurrent fractures, was caused by loss of function mutations in LRP5 [10]. Immediately after this discovery, a gain-of-function mutation in LRP5 was identified that manifests a high bone mass phenotype [11–13]. Moreover, *Lrp5* KO mice (*Lrp5*$^{-/-}$) display decreases in bone mass, mechanical properties, and mechanosensitivity [14–16].

Dickkopf-1 (DKK1) is a secreted glycoprotein, which is a potent Wnt antagonist [17] and is vital for head and limb development [17,18]. DKK1 also acts within the bone to specifically block Wnt/β-catenin signaling in osteoblasts, thereby inhibiting osteoblast development and activity [19]. DKK1 exerts this function by binding to either of the single-pass transmembrane receptor proteins Kremen 1 or Kremen 2 and also within the carboxyl-terminal propeller domains of either the LRP5 or LRP6 co-receptors [20]. It is thought that increased DKK1 levels or activity may lead to impaired osteoblast activity and bone loss [21]. In humans, unbiased global gene expression analyses have identified *DKK1* as a gene associated with bone mineral density (BMD) variation in postmenopausal Caucasian women [22], and elevated serum DKK1 concentrations were inversely associated with BMD in patients with osteoporosis [23]. In mice, the physiological role of DKK1 in the bone has been examined using the heterozygous *DKK1* KO mice [24] and doubleridge mice harboring a hypomorphic allele of *DKK1* [25]. Though complete loss of DKK1 function led to embryonic lethality [18], diminished DKK1 levels resulting from the lack of a functional *DKK1* allele

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resulted in alterations in bone development and patterning [25] and increased bone mass [24]. In contrast, overexpression of DKK1 resulted in lower BMD due to lower rates of bone formation [26–29]. Pre-clinical studies with DKK1 neutralizing antibody (DKK1-Ab) stimulated bone formation at both cortical and trabecular sites [30], increases bone mineral density in adult ovariectomy (OVX) mice [31], and promoted fracture healing and implant fixation in rodent models [30,32]. In addition, DKK1-Ab has been shown to reverse the bone destruction pattern observed in a mouse model of rheumatoid arthritis [33].

Although DKK1 is important in fracture repair, the mechanism is still unclear. Wnt pathway components (*Wnt4*, *Fzd2*, *Lrp5* and *β-catenin*) were up-regulated at the fracture site within 3–5 days after injury [34] . A recent report demonstrated that β-catenin was localized in the nuclei of several types of cells, including periosteal cells and osteoblasts near the fracture site, mature chondrocytes of the fracture callus and osteocytes at cortical bone 5 days post-fracture. Furthermore, activation of the Wnt pathway appears to improve bone healing in mesenchymal stem cells [35]. In the latter studies, Ad-DKK1 treatment of fractures resulted in a failure to heal and was associated with the accumulation of undifferentiated mesenchymal cells [35]. In the present studies we investigated if DKK1-Ab promotes fracture healing through activating β-catenin signaling in mesenchymal progenitor cells using *β-catenin*^{Prx1ER} conditional knockout (KO) mice.

Materials and method

Experimental animals

(1) 10-week-old male CD1 mice were subjected to tibial open fracture. After surgery, mice were divided into two groups: DKK1-Ab treatment group (25 mg/kg, subcutaneous injection, twice a week for 28 days); and Vehicle (PBS) control group.
(2) To generate *Prx1-CreER;β-catenin*$^{fx/fx}$ (*β-catenin*^{Prx1ER}) mice, *β-catenin*$^{fx/fx}$ mice [36] (obtained from Jackson Laboratory) were bred with *Prx1-CreER* transgenic mice [37] (obtained from Dr. Malcolm Logan, National Institute for Medical Research, London, UK). 10-week-old *β-catenin*^{Prx1ER} mice (*Prx1-CreER;β-catenin*$^{fx/fx}$) and Cre-negative control mice (*β-catenin*$^{fx/fx}$) (C57BL/6 background) were subjected to tibia fracture. Mice were treated with DKK1-Ab or Vehicle as above. Tamoxifen (TM, Sigma, St. Louis, MO) was administered immediately after fracture surgery (1 mg/10 g body weight/day, i.p. injection for 5 days). DKK1-Ab was administered after tamoxifen induction.

Mouse genotyping was determined by PCR using a DNA extraction kit (Sigma, St. Louis, MO) from tail biopsy tissues 1 month after birth. PCR primer sequences for genotyping were as follows, upper primer 5′ - AAGGTAGAGTGATGAAAGTTGTT - 3′ and lower primer 5′ - CACC ATGTCCTCTGTCTATTC - 3′ (324-base-pair PCR product). All mice had free access to food and water during entire study. All procedures conducted in this study were approved by IACUC committee of University of Rochester.

Tibial fracture model

A unilateral (right side) open transverse tibial fracture with intramedullary needle fixation was used as the bone fracture model similar to that described previously [38]. This model involves a standardized surgical approach that leads to a reproducible injury that resembles repair in human tibial fractures [38]. 10-week-old mice were anesthetized with Ketamine (60 mg/Kg) by intraperitoneal injection. A 1.5 cm-long skin incision was made along the anterior-medial surface of the shaved tibia. On the medial side of the patellar ligament, a 27 gauge syringe needle was inserted into the bone marrow cavity of the tibia through the tibial plateau. The needle was removed, and a No. 11 surgical blade was used to transect the diaphysis of the tibia at the midpoint. Either a 25 or 27 gauge needle was then inserted into the tibia to stabilize the fracture. Minimal damage was made to the adjacent regions of the periosteum during the surgical procedure. A 4-0 or 5-0 silk suture was used to close the wound and buprenorphine was administered in the drinking water for pain relief for the first three days after surgery [39].

Cre-recombination efficiency

To determine if the *Prx1-CreER* transgene could target floxed genes specifically in mesenchymal progenitor cells at the fracture site, *Prx1-CreER* transgenic mice were bred with *Rosa*^{LacZ} (*R26R*) or *Rosa*$^{mT/mG}$ reporter mice. Tamoxifen (TM, 1 mg/10 g body weight/day, i.p. injection for 5 days) was administered immediately after fracture and mice were sacrificed 5 or 10 days later for analysis. Cre-recombination efficiency was evaluated by X-gal staining. To evaluate Cre-recombination efficiency, we counted the number of X-gal positive cells and divided by total cell number in callus tissue.

Radiographic and μCT Analyses

CD1 mice, *β-catenin*^{Prx1ER} mice and Cre-negative mice were sacrificed, at days 7, 10, 14, 21 and 28 post-surgery for tissue analysis. Radiographic analysis (Faxitron X-ray, Wheeling, IL) was performed on fracture samples in both anterior–posterior and lateral orientations are performed immediately after surgery to confirm that the osteotomy was complete and pinned correctly. After mice were sacrificed, fracture healing was examined (n = 10 mice at each time point) by assessment of bridging across cortices. The extent of bridging between the fracture gap was determined qualitatively in a blinded fashion by three independent investigators using the following criteria: 1) no healing (gap present with only rudimentary evidence of repair); 2) partial healing (some gap closure with evidence of bridging); and 3) complete healing (no gap with complete bridging). Specimens were scanned at 10.5-micron isotropic resolution using a Scanco VivaCT 40 (Scanco Medical AG, Switzerland) at indicated time points. Callus total volume (TV), callus bone volume (BV), callus mineralized volume fraction (BV/TV) (%) and callus bone mineral density (BMD) were determined (n = 6 in each time point). For the CD1 mice day 28 group, some animals died or the fracture procedure failed and so radiographs, μCT and histology (below) data were not shown for this group.

Biomechanical torsion testing

Soft tissue-free full length tibia bone samples were harvested (n = 6 at days 10, 14, 21, and 28). Tissues were fixed in aluminum square tubes (0.5 cm) filled with bone cement to make sure that fracture lines were in the middle of the interval. Fracture specimens were mounted on an EnduraTec TestBench™ system with a 200 N·mm torque cell (EnduraTec TestBench™ system, Bose Corp., Minnetonka, MN) and tested in torsion at a rate of 1°/s until failure to determine the torsional stiffness and ultimate torque [39].

Quantitative gene expression analysis

The fracture callus including 1 mm of adjacent bone on either side of the fracture line was harvested and total RNA was extracted using the PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA) (n = 3 in each time point). One microgram total RNA was used to synthesize cDNA using iScripts cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time PCR amplification was performed using gene specific primers and SYBR green real-time PCR kit (Bio-Rad, Hercules, CA). The levels of the target gene expression were normalized to that of β-actin in the cDNA sample. Real-time RT-PCR analysis was performed using murine specific primers for chondrogenesis (*Sox9*, *Col2a1*, *Aggrecan*, *Col10*, *Mmp9*, *Mmp13*), osteogenesis related genes (*Runx2*, *osterix*, *osteocalcin*, *Dkk1*,