Anti-Dkk1 Antibody Promotes Bone Fracture Healing through Activation of β-Catenin Signaling
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Introduction:

Dkk1 is a Wnt signaling antagonist which binds the LRP5 receptor and blocks Wnt/LRP5 signaling in bone cells. Dkk1 over-expressing transgenic mice developed an osteopetrotic phenotype [1]. In vivo application of Dkk1 neutralizing antibody (Dkk1-Ab) stimulates bone formation, increases bone mineral density, and enhances bone fracture healing [2]. In addition, Dkk1-Ab has also been shown to reverse bone destruction pattern of a mouse model of rheumatoid arthritis to the bone-forming pattern of osteoarthritis [3].

Wnt/β-catenin is one of the critical signaling pathways that regulates chondrogenesis, osteogenesis, and osteoclast formation. Wnt pathway components (Wnt4, Fzd2, Lrp5 and β-catenin) were up-regulated at the fracture site within 3-5 days after injury [4]. A recent report demonstrated that β-catenin, histology, biomechanical testing and RNA and histological analyses near the fracture site, mature chondrocytes of the fracture callus and osteocytes at cortical bone 5 days post-fracture. Activation of Wnt pathway has potential to improve bone healing in mesenchymal stem cells [5]. In this study we hypothesize that Wnt/β-catenin signaling in mesenchymal progenitor cells might play a critical role in fracture healing process and Dkk1-Ab promotes fracture healing through activation of β-catenin signaling.

Methods:

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 PBS control group. (2) To generate Prx1CreER/β-cateninLoxPloxP (β-catenin cKO) mice, β-cateninLoxPloxP mice were bred with Prx1CreER transgenic mice. 10-week-old β-catenin cKO mice and Cre-negative mice were subjected to tibia fracture. Mice were treated with Dkk1-Ab and PBS, respectively. Tamoxifen (TM, 1mg/kg body weight/day, i.p. injection for 5 days) was administered right after fracture surgery.

Tibial Fracture Model: An intramedullary pin was inserted into the tibia at the knee and an open fracture in the proximal tibia diaphysis was performed using a No.11 scalpel blade. The fibula was left undamaged so as to produce a stabilized fracture. Specimens were harvested at days 7, 10, 14, and 21 for biomechanical testing and RNA and histological analysis.

Cre-recombination Efficiency: To determine if the Prx1CreER transgene could target floxed genes specifically in mesenchymal cell in fracture site, Prx1CreER transgenic mice were bred with Rosa26 reporter mice. Tamoxifen (TM, 1 mg/10 g body weight/day, i.p. injection for 5 days) was administered right after fracture. The mice were sacrificed at day 10. Cre-recombination efficiency was evaluated by X-Gal staining. 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) and callus bone mineral density (BMD) were determined.

Biomechanical Torsion Testing: Fracture specimens were mounted on an Enduratec TestBench 3000 with a 200 N load cell (Bose Corp., Minnetonka, MN) and tested in torsion at a rate of 1°/sec until failure to determine the torsional stiffness, ultimate torque, ultimate rotation, and strain to energy failure.

Quantitative PCR: The fracture callus and 1mm of adjacent bone was harvested and total RNA was extracted using the Qiagen RNeasy kit. cDNA was synthesized from 1µg of RNA per callus using a iScript cDNA synthesis kit (Bio-Rad). Real-time RT-PCR analysis was performed using murine specific primers for chondrogenesis and osteogenesis related genes (Sox9, col2a1, colX, Runx2, Osterix, osteocalcin, Dkk1, β-catenin).

Histology & Histomorphometry: Specimens were harvested at 7, 10, 14, 21 and 28 days, and fixed in 10% NB-Formalin for 3 days and decalcified for 14 days in 14% EDTA and then paraffin embedded. 3-µm sections were cut and prepared and Alcian blue/H&E staining was performed. Histomorphometric analysis was performed using Osteometrics software (DaContr, GA).

Statistical Analysis: Results were presented as the mean ± standard deviation. Statistical analyses included Student’s t-tests and two-way ANOVA. p<0.05 was considered as significant.

Results:

X-Gal staining showed that the Cre-recombination efficiency was 63% in the callus tissue. X-Gal positive cells include mesenchymal cells, osteoblasts, chondrocytes and bone marrow cells in the callus tissue. Radiographic and µ-CT analyses showed that administration of Dkk1-Ab enhanced bone callus formation. The fracture line was more obscure in Dkk1-Ab group at day 14 and 21. In Prx1CreER/β-cateninloxPloxP (β-catenin cKO) mice, the fracture line was easy to see in both Dkk1-Ab and PBS treated group at day 14 and day 21. µ-CT data showed a significant increase in bone volume of fracture callus in CD1 mice treated with Dkk1-Ab for 14, 21 and 28 days compared to controls. The bone volume of β-catenin cKO mice was decreased compared with Cre-negative control group in day 14, 21 and 28. Moreover, this phenotypic alteration can not be reversed by Dkk1-Ab, suggesting that Dkk1-Ab enhanced fracture healing through activation of β-catenin signaling.

Histological data showed that the progression of fracture healing was accelerated in Dkk1-Ab treated group in CD1 mice. However, less and smaller callus tissues were found in β-catenin cKO mice. Histomorphometric data demonstrated that the cartilage area was increased in CD1 mice treated with Dkk1-Ab at day 7, and the woven bone area was increased at day 14 and 21 after Dkk1-Ab treatment. There was less cartilage and woven bone areas in β-catenin cKO mice. In contrast, there was no significant difference between Dkk1-Ab and PBS treated groups in β-catenin cKO mice.

Results of biomechanical testing showed that treatment with Dkk1-Ab enhanced bone formation in CD1 mice. The maximum torque and stiffness were significantly increased at day 10, 14, 21, and 28 in Dkk1-Ab treated group compared to control group at day 7 and day 10.

Gene expression data showed that the expression of cartilage marker genes (Sox9, col2a1, and colX) was increased at day 7 in Dkk1-Ab treated group in CD1 mice. The expression of bone marker gene (Runx2, Osterix, and OC) was increased at day 14, 21, and 28 in Dkk1-Ab treated group. The expression of dkk1 was decreased at day 14, 21 and 28 and the expression of β-catenin was up-regulated at day 21 and 28 in Dkk1-Ab treated group.

Discussion

In the present studies, we demonstrated that treatment with Dkk1-Ab increased bone callus formation and mechanical strength during fracture healing in CD1 mice. Dkk1-Ab enhances fracture healing by increasing mesenchymal cell proliferation and expansion and promoting osteoblast differentiation. The fracturing healing process is delayed when the β-catenin gene is deleted in mesenchymal progenitor cells during early fracture healing stage. Dkk1-Ab seems acting through β-catenin signaling to enhance fracture healing since Dkk1-Ab lost its activity to promote fracture healing in β-catenin cKO mice. Further understanding the signaling mechanism of Dkk1-Ab in bone formation and bone regeneration will facilitate the clinical translation of this important anabolic agent into therapeutic intervention.

Significance

Our studies demonstrated that Dkk1-Ab has anabolic effect to enhance fracture healing, possibly through activation of β-catenin signaling in mesenchymal progenitor cells. Dkk1-Ab could serve as a potential clinical anabolic agent promoting fracture healing process.

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References:


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