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The effects of Dickkopf-1 antibody on metaphyseal bone and implant fixation under different loading conditions

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ABSTRACT

The secreted protein Dickkopf-1 (Dkk1) is an antagonist of canonical Wnt signaling, expressed during fracture healing. It is unclear how it is involved in the mechanical control of bone maintenance. We investigated the response to administration of a Dkk1 neutralizing antibody (Dkk1-ab) in metaphyseal bone under different loading conditions, with or without trauma. In this three part experiment, 120 rats had a screw or bone chamber inserted either unilaterally or bilaterally in the proximal tibia. Mechanical (pull-out) testing, μCT and histology were used for evaluation. The animals were injected with either 10 mg/kg Dkk1-ab or saline every 14 days for 14, 28, or 42 days. Antibody treatment increased bone formation around the screws and improved their fixation. After 28 days, the pull-out force was increased by over 100%. In cancellous bone, the bone volume fraction was increased by 50%. In some animals, one hind limb was paralyzed with Botulinum toxin A (Botox) to create a mechanically unloaded environment. This did not increase the response to antibody treatment with regard to screw fixation, but in cancellous bone, the bone volume fraction increased by 233%. Thus, the response in unloaded, untraumatized bone was proportionally larger, suggesting that Dkk1 may be up-regulated in unloaded bone. There was also an increase in thickness of the metaphyseal cortex. In bone chambers, the antibody treatment increased the bone volume fraction. The results suggest that antibodies blocking Dkk1 might be used to stimulate bone formation especially during implant fixation, fracture repair, or bone disuse. It also seems that Dkk1 is up-regulated both after metaphyseal trauma and after unloading, and that Dkk1 is involved in mechano-transduction.

Introduction

Most fractures occur in osteoporotic cancellous bone in metaphyseal regions, such as the hip, spine or forearm. Screws, pins and plates may be inserted to stabilize these fractures. In cancellous bone, the response to the trauma of inserting a screw is similar to metaphyseal fracture repair: both are examples of trauma-induced membranous bone formation. This new bone formation is important for the strength of screw fixation, especially when the initial fixation is weak. Consequently, the regeneration of bone after trauma can be estimated by measuring the mechanical fixation of screws and the formation of new bone around them. The early fixation of total joint replacements also depends on a fracture-type response in metaphyseal bone. Increased bone formation at early stages in the healing or incorporation process might provide a better long-term prognosis due to the correlation between improved early fixation and reduced risk of late loosening [1].

Dickkopf-1 (Dkk1) is a secreted glycoprotein. It is a potent Wnt antagonist [2], vital for head and limb development [2,3]. Dkk1 binds to low-density lipoprotein related proteins 4, 5 and 6 (LRP4/5/6) [4–7]. The exact mode of action is unclear, but it appears that Dkk1 directly competes with Wnt-ligand in binding to LRP6 [8] thus antagonizing Wnt signalling [9] and increasing β-catenin degradation. This β-catenin degradation blocks mesenchymal stem cell commitment for an osteogenic fate [10]. By modulating Wnt signaling, it is possible to achieve an anabolic effect in bone. Skeletal trauma causes a local increase in Wnt signaling [11] and it has been shown that the pathways involved in bone Wnt signaling are necessary for bone healing [11,12] and for bone formation in general [13–15]. Fracture repair can be influenced by blocking Dkk1 in mice [16] and a decrease in Dkk1 gene expression leads to an increase in bone mass and strength [15,17].

Sclerostin, another bone specific antagonist to Wnt signaling, has been shown to be an important mediator of the response and adaptation of bone to mechanical loading [18]. It has also been shown that Dkk1 expression is influenced by mechanical load [19], although

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not to the same degree as sclerostin. To further investigate this, we compared the response to antibodies blocking Dkk1 in traumatized and untraumatized bone, under both loaded and unloaded conditions.

This study tested three hypotheses. The first was that systemic administration of a Dkk1 neutralizing antibody increases the healing response to trauma, thereby improving the fixation of an implanted screw. The second hypothesis was that the same antibody promotes bone generation in general, leading to increased density in untraumatized bone and increased bone formation in a titanium chamber. The third was that these responses are dependent on mechanical loading.

Materials and methods

Experimental overview

The study consists of three sets of in vivo experiments. All studies involve insertion of an implant into the proximal tibia of one or both legs. A total of 120 male, 10 week old Sprague–Dawley rats (Taconic, Lille Skensved, Denmark) with a mean weight of 360 ± 30 g were used.

In the first experiment (screw fixation), we studied the response to metaphyseal trauma by inserting a stainless steel screw in the right proximal tibia of 40 rats. The fixation was evaluated by mechanical pull-out testing after either 14 or 28 days ($N = 4 \times 10$). In the second experiment, we studied the capacity for bone regeneration by inserting a titanium chamber (bone conduction chamber, BCC) at the same location in 40 rats. The bone regeneration was evaluated using histology after 28 days or μCT after 42 days ($N = 4 \times 10$). In the third experiment, we studied the role of mechanical loading, by injecting Botulinum toxin A (Botox, Allergan, Irvine, CA, USA) to paralyze the muscles in one of the hind limbs in 40 animals. Screws were inserted bilaterally in the proximal tibiae for 28 days. These were either of steel, for mechanical testing ($N = 2 \times 12$), or of PMMA for morphometry measurements by μCT ($N = 2 \times 8$).

After surgery, all rats were randomly assigned to either antibody or saline injections. Animals were euthanized using carbon dioxide after 14, 28 or 42 days and both tibias were harvested. From the animals with a titanium chamber, evaluated with μCT, the L5 vertebra was also collected.

The rats were given free access to food and water during the experiment, and were housed three per cage at 21 °C in a room with 12 h light and 12 h dark cycle. The study was approved by the Regional Ethics Committee for Animal Experiments and institutional guidelines for care and treatment of laboratory animals were followed.

Implants

Stainless steel screws were used for pull-out testing. The screw heads were designed to enable mounting in a materials testing machine. The threaded part of the screw was 2.8 mm long and 1.6 mm in diameter. To avoid metal artifacts during μCT measurements, screws of the same size were made out of polymethylmethacrylate (PMMA). Bone formation adjacent to PMMA in similar models appears histologically similar to that seen around stainless steel [20]. We have previously used PMMA screws to study bone formation in the proximity of an implant [21]. To study new bone formation, we used the bone chamber [22]. It consists of a titanium screw with a cylindrical interior space 2 mm wide and 7.5 mm long. The chamber is empty at insertion, and its bone content after a certain time will reflect the capability for new bone formation by membranous ossification.

Dkk1-antibody

A chimeric mouse/rat Dkk1 neutralizing antibody (Dkk1-ab) was provided by Lilly (Lilly Research Laboratories, Indianapolis, USA). This antibody consists of mouse variable domains fused to rat IgG1 kappa constant domains. Antibody solution, 10 mg/kg, or corresponding volume of saline was given by subcutaneous injection every 14 days, starting the day after surgery. The operators and evaluators were blinded for treatment during the course of the experiment.

Botox injection

Animals allocated for unloading were anesthetized with isoflurane and given Botox intramuscularly using an insulin-syringe in the calf (three injections of 1 U) and quadriceps femoris muscles (two injections of 1 U). The hind limb for treatment (left or right) was randomly chosen. Two days after the injection, all animals presented an obvious limp and did not bear weight on the injected limb. This condition persisted for the duration of the experiment. The animals underwent implantation surgery on the third day after the Botox injection.

Surgical procedure

The surgical procedures were identical to those explained in earlier work [21,22]. The rats were anesthetized with isoflurane and operated on under aseptic conditions. Briefly, a 1.4 mm insertion hole was hand drilled in the cancellous bone, approximately 3 mm distal to the tibial physis. A steel or PMMA screw was inserted in the hole and gently screwed in place. Animals that received a bone chamber were subjected to the same procedure, but a 3.2 mm diameter drill was used to enlarge the hole created in the medial cortex. The chamber was then screwed into the hole until its cap rested on the periosteal bone surface. In case of bilateral implants the same procedure was repeated on the other limb. The animals were fully weight bearing immediately after awakening from anesthesia. Rats received 0.007 mg of buprenorphine as post operative analgesic every 12 h for 48 h.

Mechanical evaluation

All analyses were performed by investigators who were blinded for antibody treatment. Harvested bones were kept moist by saline irrigation and all bones were tested within 1 h after harvesting. Steel screws were tested for pull-out strength in a computerized materials testing machine (100 R; DDL Inc. Eden Prairie, MN, USA), at a cross-head speed of 0.1 mm/s. These types of measurements have been described in detail earlier [23]. The machine recorded the peak force and the energy uptake until the force had dropped to 90% of maximum. It also calculated the stiffness from the slope of the force/distance curve at a point chosen manually by the investigator. The peak pull-out force was considered the primary variable.

To determine if any observed effects were due to a change in the healing response, or due to a general effect on the skeleton, the fixation of incorporated screws was compared with screws inserted at the end of the experiment. A screw was inserted post mortem in the untraumatized contra-lateral tibia, similarly as during the surgical procedure. This screw was tested for pull-out strength immediately. The same screw was used for all samples. The screw was cleaned between each animal.

Histomorphometry

Histomorphometry was conducted on the bone chamber contents 28 days after insertion, all analyses were performed by investigators who were blinded for treatment. After the tibia was harvested, the tissue contents were removed from inside the chamber, decalcified, and prepared for histology. Sections parallel to the long axis of the chamber were stained with hematoxylin and eosin.

The evaluation was done by manual point counting within an area of interest from the bottom of the chamber, i.e. the ingrowth end,