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Exogenous hedgehog antagonist delays but does not prevent fracture healing in young mice☆

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ABSTRACT

Fracture healing recapitulates many aspects of developmental osteogenesis. The hedgehog (Hh) signaling pathway, essential to skeletal development, is upregulated during fracture healing, although its importance is unclear. Our goal was to assess the functional importance of Hh signaling in endochondral fracture healing. We created closed, transverse diaphyseal femur fractures in mice, stabilized with an intramedullary pin, and administered a systemic Hh inhibitor or vehicle. Because Hh pathway activation is mediated by the receptor Smoothened (Smo), we used the Smo antagonist GDC-0449 (GDC, 50 mg/kg, twice daily) to target the pathway. First, in vehicle-treated 10-wk. female C57BL/6 mice we confirmed that Hh signaling was increased in fracture callus compared to intact bone, with >5-fold upregulation of target genes *Ptch1* and *Gli1*. Additionally, using 10-wk. male and female *Gli1* reporter mice, we saw a strong activation of the reporter in the osseous regions of the fracture callus 7–10 days after fracture. GDC treatment significantly blunted these responses, indicating effective inhibition of fracture-induced Hh signaling in bone. Moreover, microCT analysis revealed that GDC treatment significantly reduced cancellous and cortical bone volume at non-fracture sites (tibial metaphysis and diaphysis), suggesting that the drug inhibited normal bone formation. GDC treatment had a modest effect on fracture healing, with evidence of delayed callus mineralization radiographically (significantly lower Goldberg score at day 14) and by microCT (reduced callus vBMD at 14 days), and a delay in the recovery of torsional rotation to normal (elevated rotation-at-peak torque at 21 days). On the other hand, GDC treatment did not inhibit qPCR or morphological measures of chondrogenesis or angiogenesis, and did not impair the recovery of failure torque (at day 14 or 21), a measure of biomechanical competence. In summary, GDC treatment inhibited Hh signaling, which delayed but did not prevent fracture healing in young mice. We conclude that Hh signaling is strongly induced after fracture and may play a role in early callus mineralization, although it does not appear to be required for eventual healing.

1. Introduction

Fractures are one of the most prevalent medical conditions, accounting for 16% of all musculoskeletal injuries. Annually, 6.8 million cases occur, incurring approximately $100 billion in medical costs and productivity loss in the United States [1–3]. While most fractures heal without difficulty, up to 10% of fractures result in healing deficiencies [3–5]. Improved understanding of the mechanisms of fracture healing will aid development of novel therapies to reduce healing deficiencies and minimize patient morbidity.

Long bone fractures typically heal by secondary fracture healing through a cartilage template intermediate. This process recapitulates events that take place during embryonic development [6–9]. One of the signaling pathways instrumental in the growth and differentiation of bone during development is the hedgehog (Hh) pathway. Of the three Hh ligands identified in vertebrates, Indian hedgehog (Ihh) is particularly critical in skeletogenesis. *Ihh* regulates proliferation and differentiation of chondrocytes, development of perichondrial osteoblasts, and vascularization of endochondral bone [10–12]. Apart from its role in skeletal development, Hh signaling is critical to vascular development in general, acting in part through BMP and VEGF pathways [13–15]. Thus, Hh is a key regulator of several developmental processes that are also critical in fracture healing - chondrogenesis, osteogenesis and vasculo/angiogenesis [15].

Several important mediators in the Hh pathway include Patched1 (*Ptch1*), a 12-transmembrane receptor that inherently inhibits Hh signaling, Smoothened (Smo), a 7-transmembrane receptor, and Glioma-associated oncogene (*Gli1*), a terminal effector of the pathway. The

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binding of Hh ligand to *Ptch1* on the cell surface releases the baseline suppression of Smo by *Ptch1*, resulting in downstream Gli1 transcription, which leads to the activation of a signal cascade [16,17]. *Ptch1* negatively regulates Hh signaling via this feedback loop, making its expression a marker of increased Hh pathway activity [18,19]. Recent advances in targeted anticancer therapies provided an effective competitive antagonist of Smo, GDC-0449 (Vismodegib) [20–23], which was FDA-approved for clinical use for basal cell carcinoma in 2012.

Despite the known importance of Hh signaling in skeletogenesis, little is known about the role of Hh signaling in post-natal fracture healing. Studies have shown that *Ptch1* expression is upregulated during non-endochondral woven bone formation in response to stress fracture [24]. Also, endochondral ossification of unstabilized fractures in mice was associated with increased *Ihh* expression [25], and a similar study in rat femurs demonstrated expression of *Ihh* in chondrocytes and osteoblasts during fracture healing [26]. Two recent studies showed a functional role for Hh in bone healing. First, genetic inactivation of Hh in osteoblasts blunted bone matrix deposition during endochondral healing in mice [27]. Second, the use of the pharmacological Smo antagonist, GDC-0449, reduced osteogenesis and angiogenesis during stress fracture healing in rats [28]. Our goal here was to extend these previous studies and assess the functional importance of Hh signaling in endochondral fracture healing. We utilized the mouse femur fracture model to determine whether pharmacological inhibition of the Hh pathway could modulate endochondral healing. We hypothesized that Hh pathway inhibition using GDC-0449 reduces Hh signaling and delays fracture healing in young mice.

2. Methods

2.1. Wild-type mice

A total of 136 female, C57BL/6 wild-type mice (Charles River Lab, MA) were obtained at 8–9 weeks of age and housed until 10 weeks old in standard conditions (12 h light/dark cycles). The mice were randomly but equally divided into two groups of 68. The treatment group received GDC-0449 (ChemieTek, Indianapolis, IN; 50 mg/kg; 20 mg/mL dissolved in DMSO), hereafter referred to as GDC, twice daily by oral gavage (#20 gavage needle) until designated euthanasia time points. This regimen was selected based on our previous work [28] and pilot studies demonstrating suppression of *Ptch1* and *Gli1* expression in bone (confirmed in Results below). The control group received equivalent volume (2.5 mL/kg) of DMSO vehicle, hereafter referred to as Veh, twice daily by oral gavage. Treatment and vehicle animals were housed in separate cages. Access to food and water was ad libitum with unrestricted cage activity. Body weight was recorded daily. Euthanasia was performed by CO_2 asphyxiation. All protocols were approved by Animal Studies Committee at Washington University in St. Louis School of Medicine.

2.2. Cre reporter mice

A total of 19 Gli1Cre reporter mice were bred in the animal barrier and allowed to mature to 10 weeks. Mice were a cross between an Ai14 tdTomato reporter (B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J, stock 007914, from Jackson Labs, Bar Harbor, ME) and a tamoxifen-inducible Gli1Cre originally reported by Ahn and Joyner [29]. Mice were either homozygous or heterozygous for Ai14 and had a single copy of Cre (Ai14; Gli1CreERT2). In pilot studies there were no differences in tdTomato expression with between homozygous or heterozygous Ai14 mice. Activation of Gli1Cre expression was induced using tamoxifen (Tmx) chow (TD.130859, Envigo, Madison, WI) started a week prior to surgery and given until euthanasia. The mice were randomly divided into Veh with normal chow (Veh/Ctrl), Veh with Tmx (Veh/Tmx), and GDC with Tmx (GDC/Tmx) groups. The GDC and Veh were administered orally in the same manner as stated in the previous section. Mice were sacrificed 7 or 10 days after surgery and processed for calcified frozen histology. Frozen sections were counterstained with DAPI and imaged (Hamamatsu NanoZoomer HT model). The tdTomato expression was cumulative throughout the experiment and labeled any cell or progeny expressing Gli1Cre.

2.3. Fracture surgery

The morning doses of Veh and GDC were given to the 10-wk. old mice prior to surgery. Mice were sedated (1–3% isoflurane) in preparation for surgery. The right hindlimb was shaved and sterilely prepared. An incision was made over the distal femur and patella subluxated to expose the femoral condyles and release the quadriceps. With the mid-diaphysis of the femur exposed, a small notch on the medial surface was made with a #12 blade in order to pre-dispose this site to fracture. A #70 drill bit was used to open the intercondylar notch. A series of 27G, 25G, and 23G needles were used to ream the intramedullary canal. A fracture of the right femur was created using three-point bending loading, with the load applied using a materials testing machine (Instron) as described [30]. The incision was lavaged with sterile saline for better visualization of the fracture site and proximal fracture fragment. A 23G pin with flattened ends was inserted until fully seated in the intramedullary space. The pin was cut to the appropriate length and the incision was closed with 3-O nylon sutures (McKesson Medical-Surgical, Mn). Buprenorphine was injected for post-operative pain control (0.1 mL/20 g). Mice were returned to cage for recovery and allowed unrestricted cage activity. Day of surgery was defined as post-operative day zero (POD0). For all experiments the right femur (RF) was injured and the left femur (LF) was used as an uninjured internal control.

2.4. Radiographs

After the surgery, the lower extremities were radiographed at 3× magnification with the mouse placed prone and the hips abducted (Faxitron UltraFocus 100; Tucson, AZ). Radiographs were taken again in the same manner at the time of sacrifice (either POD 7, 10, 14, or 21), to ensure fracture stability and alignment during the course of treatment and to note the stage of mineralization. The radiographs (POD7–21) were independently graded in a blinded manner by two investigators using a subset of the Goldberg Scale (0 = no union; 1 = partial union; 2 = complete union) [31]. Any discrepancies were adjudicated by the senior investigator (MJG). For any mice where the radiograph displayed an absent or unstable pin (partial pullout) the femur was excluded from all analysis.

2.5. Ex vivo micro-CT of fractured femurs and intact tibias

On POD14 or 21, mice were euthanized and bilateral femurs dissected free of adjacent tissues. The femurs were placed in 10% neutral buffered formalin (NBF) overnight, washed twice with phosphate buffered saline (PBS), and placed into 70% ethanol. The proximal 2–3 mm of the fractured right femurs were potted in 2% agarose in a 21 mm diameter tube and the entire femur scanned in air using a microCT (VivaCT, Scanco Medical AG, Switzerland). Scan settings were 21 μm voxel size, 55 kVp, 145 μA, and 300 ms integration time. Images were filtered (gauss sigma 1.2) and the fracture midpoint was identified. The region of interest (ROI) was defined as 300 slices (6.3 mm, encompassing the entire callus) centered at the fracture midpoint. Contours marking the outer margin of the callus were drawn. An upper threshold of 460/1000 (16 bit grey scale normalized to 1000; equal to 840 mgHA/cm^3) was used to segment only cortical bone, while a lower threshold of 150/1000 (16 bit grey scale normalized to 1000 157.4 mgHA/cm^3 was used to segment any mineralized tissue. Total tissue volume (TV, mm^3), bone volume (BV, mm^3), bone volume fraction (BV/TV), tissue mineral density (including cortical bone) (TMD, mg HA/cm^3), volumetric bone mineral density of callus (excluding cortical bone) (vBMD, mg HA/cm^3), and callus volume (=TV - cortical bone, mm^3) were analyzed according to previously published methods [30]. Following microCT, femurs were decalcified and processed for histology.