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Hedgehog signaling mediates woven bone formation and vascularization during stress fracture healing☆

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ABSTRACT

Hedgehog (Hh) signaling is critical in developmental osteogenesis, and recent studies suggest it may also play a role in regulating osteogenic gene expression in the post-natal setting. However, there is a void of studies directly assessing the effect of Hh inhibition on post-natal osteogenesis. This study utilized a cyclic loading-induced ulnar stress fracture model to evaluate the hypothesis that Hh signaling contributes to osteogenesis and angiogenesis during stress fracture healing. Immediately prior to loading, adult rats were given GDC-0449 (Vismodegib — a selective Hh pathway inhibitor; 50 mg/kg orally twice daily), or vehicle. Hh signaling was upregulated in response to stress fracture at 3 days (Ptch1, Gli1 expression), and was markedly inhibited by GDC-0449 at 1 day and 3 days in the loaded and non-loaded ulnae. GDC-0449 did not affect Hh ligand expression (Shh, Ihh, Dhh) at 1 day, but decreased Shh expression by 37% at 3 days. GDC-0449 decreased woven bone volume (−37%) and mineral density (−17%) at 7 days. Dynamic histomorphometry revealed that the 7 day callus was composed predominantly of woven bone in both groups. The observed reduction in woven bone occurred concomitantly with decreased expression of Alpl and Ibsp, but was not associated with differences in early cellular proliferation (as determined by callus PCNA staining at 3 days), osteoblastic differentiation (Osx expression at 1 day and 3 days), chondrogenic gene expression (Acan, Sox9, and Col2α1 expression at 1 day and 3 days), or bone resorption metrics (callus TRAP staining at 3 days, Rankl and Opg expression at 1 day and 3 days). To evaluate angiogenesis, vWF immunohistochemistry showed that GDC-0449 reduced fracture callus blood vessel density by 55% at 3 days, which was associated with increased Hif1α gene expression (+30%). Dynamic histomorphometric analysis demonstrated that GDC-0449 also inhibited lamellar bone formation. Lamellar bone analysis of the loaded limb (directly adjacent to the woven bone callus) showed that GDC-0449 significantly decreased mineral apposition rate (MAR) and bone formation rate (BFR/BS) (−17% and −20%, respectively). Lamellar BFR/BS in the non-loaded ulna was also significantly decreased (−37%), indicating that Hh signaling was required for normal bone modeling. In conclusion, Hh signaling plays an important role in post-natal osteogenesis in the setting of stress fracture healing, mediating its effects directly through regulation of bone formation and angiogenesis.

1. Introduction

Multiple clinically relevant scenarios spanning virtually all orthopedic subspecialties involve osteogenesis, including spinal fusion, distraction osteogenesis, physiologic anabolism secondary to load-bearing exercises, and fracture healing. The sheer number of orthopedic patients affected by conditions requiring bone healing, the associated treatment and recovery process, and potential complications, all place a significant burden on society in terms of patient morbidity and loss of productivity. Therefore, further elucidation of the pathways mediating osteogenesis in the adult skeleton may provide an opportunity to develop novel therapeutic strategies that enhance bone healing or prevent skeletal fragility.

Hedgehog (Hh) proteins are fundamental to animal development and are conserved in species ranging from *Drosophila melanogaster* to humans [1,2]. The mammalian family of Hh proteins includes Sonic hedgehog (Shh), Desert hedgehog (Dhh), and Indian hedgehog (Ihh). These ligands signal through a mechanism involving two transmembrane proteins: Patched homolog 1 (Ptch1) and Smoothened (Smo) [3]. Hh binding to Ptch1 on the cell surface relieves the inhibition of Smo and activates an intracellular signaling cascade, resulting in increased transcription of downstream genes including glioma-associated oncogene-1 (Gli1) [4,5], Hedgehog-interacting protein-1 (Hip1) [6], and Ptch1 itself. Thus, Ptch1 is a negative regulator of Hh

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signaling through this negative feedback loop, and increased Ptch1 expression is a marker of increased Hh pathway activation [7,8]. Exogenous modulators of the pathway include Hh inhibitor GDC-0449 (Vismodegib) that acts directly on Smo [8–10]. The mechanism of action, pharmacokinetics, and pharmacodynamics of GDC-0449 have been studied extensively, mostly in the setting of cancer [9–13].

The Hh pathway is known to play a critical regulatory role in the context of embryonic limb patterning [14–17] and osteogenesis [18–21]. Ihh is required for development of perichondrial osteoblasts and vascularization of endochondral bone [14,21], indicating that Hh signaling is key to the coupling of osteogenesis and angiogenesis during skeletal development.

The role of Hh signaling in post-natal osteogenesis is poorly understood especially in the context of bone healing. Upgregulated Hh signaling was observed during healing of murine rib fractures [22] and rat ulna stress fractures [23], and Ihh expression was seen with healing of fractures [24,25]. However, it remains unclear whether these observations represent a causal mechanism, or merely correlation. A recent study not only confirmed increased expression of Hh pathway components after fracture (stabilized murine tibial fractures), but also showed that both ubiquitous and osteoblast-specific Hh activation increased fracture callus matrix deposition (%BV/TV) [26]. Osteoblast-specific Hh inhibition, but not ubiquitous inhibition, led to a statistically significant decrease in fracture callus volume as compared to controls [26]. However, the mechanism responsible for these findings is yet to be elucidated.

In combination, these studies [18,19,22–26] provide support for the role of Hh signaling in endochondral bone repair. Our goal was to extend these findings in two novel ways. First, we utilized the systemic Hh antagonist GDC-0449 to determine whether pharmacological manipulation of the pathway affected bone repair. Second, we assessed the role of Hh in stress fracture healing, which is predominantly a non-endochondral repair process. We hypothesized that the woven bone healing response to stress fracture is a Hh-dependent process. Additionally, we hypothesized that the angiogenic response to stress fracture is also Hh-dependent. In order to investigate these hypotheses, we used a previously-described fatigue loading protocol to create mid-diaphyseal stress fractures in adult rat ulnae [23,27,28]. The experimental group was treated with the Hh inhibitor GDC-0449 and compared to a vehicle group. Differences were examined using quantitative real-time PCR (qRT-PCR), microCT, immunohistochemistry and dynamic histomorphometry. Our results demonstrate that Hh signaling contributes to the osteogenic and angiogenic response during stress fracture healing.

2. Materials and methods

2.1. Animals

A total of 70 male Fischer F344 rats (Harlan) were obtained at 13–14 weeks of age and housed until ready for mechanical loading at 18–22 weeks of age (340 ± 30 g). After mild sedation with isoflurane (1–3%), treatment group rats were administered an initial dose of GDC-0449 (50 mg/kg; 50 mg/ml solution in DMSO) immediately prior to mechanical loading that was continued twice daily by oral gavage until sacrifice. Using the identical dosing schedule, control group rats received an equivalent volume of DMSO vehicle (1 ml/kg twice daily by oral gavage). Treatment and vehicle animals were housed separately. Access to chow and water was ad libitum, and cage activity was unrestricted. Rat weight was recorded daily. All protocols were approved by the Animal Studies Committee at Washington University in St. Louis.

2.2. Mechanical loading

Rats were anesthetized (1–3% isoflurane). Previously-described fatigue-loading methods were used to produce a right ulna (RU) mid-diaphyseal stress fracture (time point defined as 0 days) [23,28,29]. To summarize, the right ulna olecranon process and flexed carpus were placed in custom fixtures of a servohydraulic materials testing system (Instron 8841). A 0.3 N compressive preload was applied, followed by an axial 18 N, 2 Hz haversine waveform until a peak displacement of 1.3 mm occurred relative to the 10th cycle (equivalent to 65% of the average total displacement to full fracture). These loading parameters consistently produced a non-displaced, oblique fatigue/stress fracture on the concave (compression) surface of the ulna, which was followed by a woven bone healing response histologically similar to intramembranous fracture repair [28,29]. Buprenorphine (0.05 mg/kg intramuscular) was provided for analgesia. The contralateral left ulna (LU) served as an internal non-loaded control. Rats were returned to their cages, recovered from anesthesia, and were able to ambulate without complications.

2.3. Gene expression

After mechanical loading, rats were euthanized with CO_2 asphyxiation at 1 day or 3 days (28 rats total; n = 6–7/group/time point) for gene expression using qRT-PCR as previously described [23]. Bilateral ulnae were immediately dissected without disrupting the periosteum, and frozen in liquid nitrogen. Each ulna was processed individually. The central 5 mm of each ulna (containing the stress fracture site and callus) was isolated and pulverized. Each sample was reconstituted in TRIzol (Ambion). Chloroform was added, and the nucleic acid phase was isolated using phase lock gel tubes (Eppendorf). Total RNA was isolated using an RNeasy mini kit (Qiagen) and DNase I (Qiagen) was applied, as per manufacturer's instructions. RNA concentration was quantified (ND-1000, Nanodrop), and RNA integrity was evaluated (Bioanalyzer 2100, Agilent Technologies). Before proceeding, samples were required to meet quality standards including 260/280 nm absorbance ratios of 1.8–2.1 (Nanodrop) and electrophoretograms consistent with RNA integrity numbers (RIN) ≥7 (Bioanalyzer 2100). First strand cDNA was produced (iScript, Biorad) from 500 ng of total RNA. qRT-PCR was conducted in triplicate with a reaction volume of 20 μl using Power SYBR green detection (StepOnePlus™, Applied Biosystems). A total of 17 target genes were evaluated: three Hh pathway mediators — Gli1, Ptch1, and Hhip; three Hh ligands — Shh, Ihh, and Dhh; three osteogenic genes — alkaline phosphatase liver/bone/kidney [Alpl], osterix [Osx], and bone sialoprotein [Ibsp]; three chondrogenic genes — aggrecan [Acan], sex determining region Y-box 9 [Sox9], and collagen type 2-α1 [Col2α1]; two angiogenic genes — hypoxia-inducible factor 1 [Hif1α], and platelet/endothelial cell adhesion molecule-1 [Pecam1]; two osteoclastogenesis regulatory genes — receptor activator of nuclear factor κB ligand [Tnfsf11 (Rankl)], and osteoprotegerin [Tnfrsf11b (Opg)]; and one validated reference gene — pumilio homolog 1 [Pum1]. Primers were either previously validated (Bsp, Osx, Ptch1, Pecam1, Hif1a) [23,29], or purchased as predesigned sets and validated prior to use (QuantiTect Primer Assays, Qiagen [Pum1:QT01607396, Gli1:QT01586662, Hhip:QT01566663, Ihh:QT01618113, Dhh:QT01593697] or PrimeTime qPCR primers, IDT [Shh:Rn.PT.53a.8183165, Sox9:Rn.PT.53a.29440750, Col2a1:Rn.PT.53a.13642555, Alpl:Rn.PT.53a.10731288, Acan:Rn.PT.53a.9306870], Tnfsf11:Rn.PT.58.9292526, Tnfrsf11b: Rn.PT.58.11476004). Relative quantification of gene expression was examined after first normalizing to Pum1 ($2^{-\Delta Cq}$); in some cases expression was further examined after normalizing loaded RU relative to the non-loaded LU ($2^{-\Delta\Delta Cq}$).

2.4. Micro-computed tomography (microCT)

Right forelimbs (n = 13–14/group) underwent microCT imaging as previously described 7 days after mechanical loading (mCT40, Scanco Medical; 55 kV, 200 ms, 16 μm resolution) [23,27,28]. The central 14 mm of each ulna containing the entire fracture callus was chosen as the region of interest. The manufacturer's software (μCT v6.0) was used to determine longitudinal crack extent by measuring the distance