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Metaphyseal and diaphyseal bone loss in the tibia following transient muscle paralysis are spatiotemporally distinct resorption events

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ABSTRACT

When the skeleton is catabolically challenged, there is great variability in the timing and extent of bone resorption observed at cancellous and cortical bone sites. It remains unclear whether this resorptive heterogeneity, which is often evident within a single bone, arises from increased permissiveness of specific sites to bone resorption or localized resorptive events of varied robustness. To explore this question, we used the mouse model of calf paralysis induced bone loss, which results in metaphyseal and diaphyseal bone resorption of different timing and magnitude. Given this phenotypic pattern of resorption, we hypothesized that bone loss in the proximal tibia metaphysis and diaphysis occurs through resorption events that are spatially and temporally distinct. To test this hypothesis, we undertook three complimentary in vivo/μCT imaging studies. Specifically, we defined spatiotemporal variations in endocortical bone resorption during the 3 weeks following calf paralysis, applied a novel image registration approach to determine the location where bone resorption initiates within the proximal tibia metaphysis, and explored the role of varied basal osteoclast activity on the magnitude of bone loss initiation in the metaphysis using μCT based bone resorption parameters. A differential response of metaphyseal and diaphyseal bone resorption was observed throughout each study. Acute endocortical bone loss following muscle paralysis occurred almost exclusively within the metaphyseal compartment (96.5% of total endocortical bone loss within 6 days). Using our trabecular image registration approach, we further resolved the initiation of metaphyseal bone loss to a focused region of significant basal osteoclast function (0.03 mm^3) adjacent to the growth plate. This correlative observation of paralysis induced bone loss mediated by basal growth plate cell dynamics was supported by the acute metaphyseal osteoclastic response of 5-week vs. 13-month-old mice. Specifically, μCT based bone resorption rates normalized to initial trabecular surface (BRR_{BS}) were 3.7-fold greater in young vs. aged mice (2.27 ± 0.27 $\mu m^3/\mu m^2$/day vs. 0.60 ± 0.44 $\mu m^3/\mu m^2$/day). In contrast to the focused bone loss initiation in the metaphysis, diaphyseal bone loss initiated homogeneously throughout the long axis of the tibia predominantly in the second week following paralysis (81.3% of diaphyseal endocortical expansion between days 6 and 13). The timing and homogenous nature are consistent with de novo osteoclastogenesis mediating the diaphyseal resorption. Taken together, our data suggests that tibial metaphyseal and diaphyseal bone loss induced by transient calf paralysis are spatially and temporally discrete events. In a broader context, these findings are an essential first step toward clarifying the timing and origins of multiple resorptive events that would require targeting to fully inhibit bone loss following neuromuscular trauma.

Introduction

Profound catabolic alterations in skeletal morphology are induced by numerous exogenous and endogenous stimuli [1–6]. Induced bone loss ranges in extent from focal bone resorption caused by localized deficits in the skeletal loading environment [7,8] to broad or systemic osteopenia, such as what occurs following ovariectomy [9,10], bed-rest [11,12], or as a complication of chronic inflammatory diseases [13,14]. Given this variability and the differing permissiveness to resorption of trabecular and cortical bone, it has proven difficult to identify common cellular mechanisms that account for such a wide range of resorptive outcomes.

In this context, heterogeneous bone resorption (both between and within individual bones) has been identified in a variety of induced bone loss models [15,16]. In these models, trabecular bone has been observed to be more susceptible to osteoclastic resorption than cortical bone [16–19]. However, both genetic (e.g., gender and genetic background) and non-genetic (age and skeletal location/time period investigated) factors can alter, and in some cases transpose, this generality [20–23]. Cancellous and cortical bone alterations have, as a result, predominantly been studied as independent outcomes. A more complete understanding of how bone loss initiates, propagates and interacts among and between distinct skeletal structures would be an important

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first step toward understanding the variability of cellular dynamics that may underlie the range of tissue-level resorption in these models.

We have previously demonstrated that transient muscle paralysis of the murine calf muscles induces rapid and profound osteoclastic bone resorption in both trabecular and cortical compartments within the tibia adjacent to the paralyzed muscles [24]. Furthermore, we have previously characterized the temporal nature of this bone loss through acute time course μCT imaging of trabecular bone (proximal tibia metaphysis) and cortical bone (tibia diaphysis) resorption and have found that the timing and magnitude of bone resorption differ greatly in these compartmental tissues [25]. Specifically, significant trabecular resorption in the metaphysis is first observed 3 days following muscle paralysis [25]. Cortical resorption within the tibia diaphysis was slower to occur, with endocortical expansion (as a surrogate for endocortical resorption) first identified 9 days later [25]. While these μCT measures may suggest that temporally distinct bone loss occurred between trabecular and cortical compartments in the model, the experimental design does not allow for the determination of whether this arose through distinct cellular events of varying robustness in the spatially disparate metaphyseal and diaphyseal compartments or whether this was due to a single continuum of osteoclast resorption initiating in the metaphysis and migrating between compartments. The distinction between a single prolonged or multiple temporally and spatially separate resorptive responses following acute muscle paralysis would be an essential first step toward clarifying the signaling pathway(s) underlying such rapid and profound osteoclastogenesis. Specifically, multiple resorptive responses would be indicative of multiple signaling pathways that would each require individual targeting to completely inhibit bone loss.

In part due to our limited understanding of resorption kinetics in our model, we recently developed a novel μCT image registration approach capable of identifying focal osteoclastic resorption in the tibia diaphysis following muscle paralysis [26]. This goal was achieved by identifying a temporally unaltered image registration landmark in the tibia that enabled accurate superpositioning of serial μCT diaphyseal volumes obtained up to 21 days apart. Utilizing this approach, we were able to quantify, with high precision, the complex spatial dynamics of endocortical osteoclast activity within the diaphyseal region. As this technique is amenable to use in any bone compartment in which a consistent image registration volume can be identified, we anticipated that with modifications to facilitate trabecular alignment this approach could be used to detect the initiating site(s) and progression of focal bone loss (if any) between the metaphysis and diaphysis of the tibia following muscle paralysis.

Given the differences in timing and magnitude of acute bone resorption in the tibia metaphysis and diaphysis following muscle paralysis and the spatial distance between these compartments, we hypothesized that bone loss in the proximal tibia metaphysis and diaphysis occurs through spatially and temporally distinct resorption events. To test this hypothesis, we first implemented an imaging strategy that allowed for spatiotemporal assessment of bone loss following muscle paralysis throughout the metaphyseal and diaphyseal compartments. These data indicated that focal endocortical bone loss initiation occurred immediately adjacent to the proximal growth plate and that metaphyseal and diaphyseal bone loss were likely to occur through distinct osteoclastic events. Two follow-up in vivo studies were used to clarify these observations. In the first, we were able to co-localize metaphyseal bone loss initiation to the location of high basal osteoclast activity within the trabecular bone of the proximal metaphysis. The final study then examined whether the level of basal osteoclastic activity influenced the magnitude of acute bone resorption following muscle paralysis.

Methods

Transient muscle paralysis model

Studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee, University of Washington. All mice were female C57Bl/6 obtained from Jackson Laboratories. Transient muscle paralysis was induced in the right calf muscle group of mice via a single injection of botulinum neurotoxin A (BTxA; 2 U/100 g body weight, Allergan Inc) [25]. This intervention induces temporally distinct degradation of trabecular bone tissue in the metaphyseal and cortical bone tissue in the diaphyseal compartments [24,25]. Mice received BTxA treatment on day 0 and were allowed free cage activity for the remainder of each experiment. Calf paralysis was confirmed 24 h post-injection by visual examination of reduced toe extension and ankle plantarflexion in the affected limb [24].

Study design

Study 1: spatiotemporal tracking of bone loss

The purpose of this study was twofold. The first was to determine if the acute bone loss initiation previously identified in the trabecular bone of the proximal tibia metaphysis [24,25] was due to trabecular permissiveness to osteoclast resorption or due to a more acute osteoclastic response in the metaphysis compared to the diaphysis. The second was to determine whether a continuum of osteoclastic resorption temporally and spatially migrates from the metaphysis to the diaphysis or occurs in two distinct events as hypothesized. For this experiment, a group of 16 week old mice ($n = 6$) received BTxA injection in the right calf immediately following a single μCT scan that encompassed both the proximal tibia metaphyseal and diaphyseal envelopes (day 0). Mice in this group were subsequently imaged on days 6, 13 and 20, and euthanized immediately following the last scan.

Study 2: spatial initiation of trabecular bone loss

The second study investigated the timing and location of bone loss initiation identified within the metaphyseal compartment in the previous experiment. Based on preliminary data we specifically sought to define the spatial location of bone resorption initiation in the highly metabolically active trabecular tissue within the proximal tibia metaphysis. Prior to the experiment, we first assessed the accuracy and precision of our image registration approach. For this, untreated mice (registration control, $n = 6$, 16 weeks) received a set of non-contiguous μCT scans of the proximal tibia metaphysis and the tibia mid-diaphysis. Mice were then removed and re-inserted into the μCT scanner and a second identical set of scans was obtained. This group of mice was used to determine the resolution at which focal bone loss initiation can be identified in the trabecular compartment of the metaphysis. Next, two groups of mice ($n = 6$/gp, 16 weeks) received intramuscular injections of either BTxA (BTxA registration group) or an equal volume of saline (saline registration group) in the right calf on day 0 followed directly by metaphyseal and diaphyseal μCT scans. As we have previously shown that a saline injection in the calf does not alter muscle, trabecular or cortical bone morphology as assessed by μCT [24], saline injected mice were used as controls to maintain consistency vs. BTxA groups (e.g., stress of injections and mouse handling). Mice were subsequently scanned on day 2 and day 3.

Study 3: basal cell activity modulates bone loss initiation

The final study sought to determine if baseline growth plate cellular dynamics alters the magnitude of acute bone loss initiation following muscle paralysis. A group of 5-week old (young, $n = 6$) and 13-month old (aged, $n = 6$) mice received a BTxA injection in their right calf followed immediately by a μCT scan of their right proximal tibia metaphysis on day 0 and a follow-up scan on day 3. The end point of this experiment was chosen based on previous studies with this model [25].

μCT imaging and segmentation

High-resolution μCT images of the right tibia were obtained from all mice on day 0 (Scanco μCT 40; 10.5 μm voxel size, 55 kVp, 145 μA).