Published in final edited form as:

Bone. 2011 February 1; 48(2): 250–258. doi:10.1016/j.bone.2010.09.005.

Comparing histological, vascular and molecular responses associated with woven and lamellar bone formation induced by mechanical loading in the rat ulna

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Abstract

Osteogenesis occurs by formation of woven or lamellar bone. Little is known about the molecular regulation of these two distinct processes. We stimulated periosteal bone formation at the ulnar mid-diaphysis of adult rats using a single bout of forelimb compression. We hypothesized that loading that stimulates woven bone formation induces higher over-expression of genes associated with cell proliferation, angiogenesis and osteogenesis compared to loading that stimulates lamellar bone formation. We first confirmed that a single bout of 100 cycles of loading using either a rest-inserted (0.1 Hz) or haversine (2 Hz) waveform (15 N peak force) was non-damaging and increased lamellar bone formation (LBF loading). Woven bone formation (WBF loading) was stimulated using a previously described, damaging fatigue loading protocol (2 Hz, 1.3 mm disp., 18 N peak force). There were dramatic differences in gene expression levels (based on qRT-PCR) between loading protocols that produced woven and lamellar bone. In contrast, gene expression levels were not different between LBF loading protocols using a rest-inserted or haversine waveform. Cell proliferation markers *Hist4* and *Ccnd1* were strongly upregulated (5- to 17-fold) 1 and 3 days after WBF loading, prior to woven bone formation, but not after LBF loading. The angiogenic genes *Vegf* and *Hif1a* were upregulated within 1 hr after WBF loading and were strongly up on days 1-3 (3- to 15-fold). In sharp contrast, we observed only a modest increase (< 2-fold) in *Vegfa* and *Hif1a* expression on day 3 following LBF loading. Consistent with these relative differences in gene expression, vascular perfusion 3 days after loading revealed significant increases in vessel number and volume following WBF loading, but not after LBF loading. Lastly, bone formation markers (*Runx2*, *Osx*, *Bsp*) were more strongly upregulated for woven (4- to 89-fold) than for lamellar bone (2-fold), consistent with the differences in new bone volume observed 10 days after loading. In summary, robust early increases both molecularly and histologically for cell proliferation and angiogenesis precede woven bone formation, whereas lamellar bone formation is associated with only a modest upregulation of molecular signals at later timepoints.

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The authors have no conflicts of interest

Keywords

rat ulna; mechanical loading; woven bone; lamellar bone; angiogenesis

Introduction

Osteogenesis can occur through formation of woven or lamellar bone. In the post-natal skeleton, woven bone forms under conditions that require rapid mineral deposition such as in distraction osteogenesis, fracture healing and stress fracture repair [1-4]. Histologically, woven bone is characterized by relatively poor organization, low mineral density and high cellularity [2]. By contrast, lamellar bone forms at a slower rate during normal skeletal growth and in response to mild anabolic stimuli. It is more highly organized, denser and has fewer cells compared to woven bone. The underlying mechanisms that differentiate woven versus lamellar bone formation have been addressed by several authors [5-8], but remain poorly understood. Because in vivo mechanical loading can stimulate either woven or lamellar bone formation [6], it provides an experimental approach to study the processes that lead to these two distinct types of bone.

Mechanical loading that generates hyperphysiological bone strain and/or damage leads to woven bone formation [9]. Fatigue loading of the rat forelimb has been used to generate bone damage resulting in an ulnar stress fracture. After a single bout of damaging fatigue loading, there is a robust repair response characterized by periosteal woven bone formation at the stress fracture site [4,10-11]. Prior to woven bone formation there is an increase in expression of genes related to cell proliferation, angiogenesis, osteogenesis and bone remodeling [12-13]. Other models of woven bone formation have also reported increased proliferation [14-15], vascularity [16-19], and osteogenic gene expression [15,20].

Mechanical loading that generates modest levels of bone strain without creating damage can stimulate lamellar bone formation [6]. The cell and molecular responses that precede lamellar bone formation have not been extensively studied, although some work has been done. Cell proliferation was increased three days after stimulation of endocortical lamellar bone formation using tibial bending [21]. Increases in angiogenic and osteogenic gene expression were reported after four days of tibial bending in mice [22]. However, it was not clear whether the loading protocol induced lamellar or a combination of woven and lamellar bone formation, because tibial bending protocols often form lamellar bone only on the endosteal surface, with woven bone on the periosteal surface [6,23-24]. To better characterize the cell and molecular changes associated with lamellar bone formation requires a loading protocol that does not induce woven bone formation. Forelimb compression and tibial bending protocols that stimulate lamellar bone formation have been reported [23,25-27], however these protocols typically involve multiple days of loading. A limitation of multi-day protocols for studies of gene expression is that events triggered by the first bout of loading cannot be separated from events triggered by subsequent bouts.

The first goal of our study was to establish a loading protocol that stimulated *only* lamellar bone formation. The lamellar loading protocol (LBF loading) needed to satisfy the following requirements: 1) loading in a single bout, 2) non-damaging, 3) no woven bone formation, and 4) a significant increase in lamellar bone formation on the loaded ulna compared to control. Our second goal was to compare the formation of lamellar bone induced by this protocol to the formation of woven bone induced by the previously established damaging fatigue protocol (WBF loading) [28]. We hypothesized that stimulation of woven bone formation (using WBF loading) induces higher over-expression of genes associated with cell

proliferation, angiogenesis and osteogenesis compared to stimulation of lamellar bone (using LBF loading).

Methods

The right forelimbs of 170 adult male rats (F344, Harlan) age 4 ½ - 5 ½ mo (340 ± 25 g), were loaded in axial compression using protocols chosen to stimulate woven or lamellar bone formation at the ulnar mid-diaphysis (Tables 1-3). Contralateral left forelimbs served as non-loaded controls. With the rats anesthetized (1-3% isoflurane), right forelimbs were positioned in a loading fixture connected to a servohydraulic materials testing machine (Instron 8841) and a 0.3 N compressive pre-load applied. Rats designated for woven bone formation (WBF) loading ($n=32$) had the right forelimbs cyclically loaded (2 Hz, 18 N) in a single bout to a total displacement increase of 1.3 mm (avg. cycles 6777 ± 6404 ; min. 469; max. 23463). The WBF loading protocol has been shown to cause an average decrease of 70% in ulnar stiffness immediately after loading [11] and to induce abundant woven bone formation 7 days later [28]. Two additional loading protocols were used to stimulate lamellar bone formation as described below. After loading the rats received analgesia (i.m., 0.05 mg/kg buprenorphine) and were returned to their cages. All rats recovered well from anesthesia and mechanical loading. They were allowed normal cage activity and ad libitum access to water and chow. Rats were euthanized by carbon dioxide asphyxia on day 0 (1 hr), day 1, day 3, or day 10 after loading. Right and left ulnae were dissected. This study was approved by our animal studies committee.

Development of two single-bout loading protocols to stimulate lamellar bone formation

In a preliminary experiment, we investigated two commonly used waveforms and a range of peak forces from 14-18 N (1900-2400 microstrain [11]) (Table 1). Our goal was to determine suitable single-bout loading protocols that did not cause bone damage and that stimulated only lamellar bone formation. The first waveform was a rest-inserted trapezoid [29]. In this waveform the load/unload period is the same as in the fatigue waveform (0.5 s), but with a 9.5 s delay between loading cycles. The second waveform was a 2 Hz haversine, the same as used in WBF loading. Five of the loading protocols were rejected because woven bone was observed on at least one histological section. There were four loading protocols that did not result in woven bone formation; we selected two for further characterization. First, we chose the 100 cycle, 15 N rest-inserted waveform (“LBF loading, rest-inserted”) due to its low total displacement. A second protocol (100 cycle, 15 N haversine waveform, “LBF loading, haversine”) was then investigated to determine whether or not differences in the loading waveform affected gene expression.

To further characterize these two protocols, we assessed the effects of loading on mechanical properties, bone damage and bone formation. The right forelimbs of 45 rats were loaded in cyclic compression for a single bout of 100 cycles using either the 15 N rest-inserted or haversine waveform (Table 2). Fifteen rats were euthanized immediately after loading and designated for three-point bending tests (Instron 8841). Ulnae were positioned on supports 15 mm apart and a displacement ramp (0.5 mm/s) was applied on the medial surface at the midpoint. Structural properties were determined from force-displacement curves (Labview 7.0). Seven rats were euthanized immediately after loading to assess damage with basic fuchsin staining [30]. Briefly, upon dissection ulnae were placed into 10% formalin for 24 hours. Bones were then subjected to graded fuchsin (80%, 90% and 100%) in a vacuum for 2 hr intervals before being embedded in plastic. Forelimbs were sectioned 1 mm distal to the midpoint (100 μ m thick) and imaged using a confocal microscope (Carl Zeiss, LSM 510). Twenty-three rats were euthanized on day 10 to measure bone formation using dynamic histomorphometry. An additional set of six “normal” rats were not loaded, but processed for dynamic histomorphometry to assess the possible

systemic effects of loading [31]. Rats were given intraperitoneal injections of calcein green (5 mg/kg, Sigma) on day 3 and alizarin-complexone (30 mg/kg, Sigma) on day 8 before being euthanized on day 10. Ulnae were then embedded in methylmethacrylate (Sigma) using standard procedures. Sections (100 μ m thick) were cut 1 mm distal to the midpoint (D1) (Leica Microsystems, SP 1600). Each section was mounted on a glass slide and imaged on a microscope (Olympus, DP-30) using fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate filters for calcein and alizarin, respectively. The sections were analyzed (Bioquant) for bone area and periosteal bone surface (BS), mineralizing surface (MS), bone formation rate (BFR) and mineral apposition rate (MAR).

Gene Expression

Forelimbs were loaded using one of three single-bout protocols to stimulate woven or lamellar bone formation: 1) WBF loading; 2) LBF loading, rest-inserted; 3) LBF loading, haversine (51 rats total; n=5-6 rats/loading group/timepoint). Rats were euthanized on day 0 (1 hr), day 1 or day 3 for assessment of ulnar gene expression using quantitative real time PCR (qRT-PCR) as described [12]. Both the loaded (right) and non-loaded (left) ulnae were frozen in liquid nitrogen immediately upon dissection. A central 5 mm piece of each ulna was pulverized, suspended in trizol, and mixed with chloroform before separating the nucleic acid phase (phase lock gel tube, Eppendorf). The total RNA was isolated using an RNeasy mini kit (Qiagen). RNA integrity was quantified (Nanodrop, ND-1000) and verified (Agilent 2100 bioanalyzer). First strand cDNA was synthesized (Superscript III, Invitrogen) from total RNA (500 ng). qRT-PCR reactions were carried out at 20 μ L total volume and measured with *Power SYBR® green* (7300 Real-Time PCR System, Applied Biosystems). Samples were run in triplicate and the average was used for further analysis. Primers were validated previously [12] or purchased as predesigned sets (QuantiTect Primer Assays, Qiagen). Eleven target genes were evaluated including histone cluster 1, H4b (*Hist4*; marker for cell proliferation), cyclin D1 (*Ccnd1*; cell cycle protein); vascular endothelial growth factor A (*Vegf*; angiogenic morphogen), hypoxia-inducible factor 1, alpha subunit (*Hif1a*; hypoxia marker), platelet/endothelial cell adhesion molecule 1 (*Pecam*; endothelial cell marker), bone morphogenetic protein 2 (*Bmp2*; osteogenesis-angiogenesis coupling factor), bone sialoprotein (*Bsp*; matrix glycoprotein mediating cell adhesion), runt related transcription factor 2 (*Runx2*; required for osteoblast differentiation), bone gamma-carboxyglutamate (gla) protein (osteocalcin) (*Bglap*; marker for fully differentiated osteoblasts), osterix (*Osx*; required for osteoblast formation), and patched homolog 1 (*Ptch*; marker of activated hedgehog pathway). Data were analyzed using relative quantification ($2^{-\Delta\Delta C_T}$) comparing loaded to non-loaded ulnae after first normalizing to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

Immunohistochemistry

BMP2, HIF1a and proliferating cell nuclear antigen (PCNA) proteins were localized using immunohistochemistry at days 1 and 3 (15 rats total; n=3-4/loading group/timepoint). Comparisons were made between WBF loading and rest-inserted LBF loading (only one LBF loading protocol was used given the similarities in results for protocol development and gene expression presented below). Following dissection, forelimbs (ulna and radius with some surrounding muscle) were dissected, fixed overnight in 4% paraformaldehyde at room temperature and decalcified in 14% EDTA. Specimens were embedded in paraffin and 5 μ m sections were cut 1 mm distal to the midpoint. Primary antibodies for BMP2 (Santa Cruz, sc-6895) and HIF1a (Santa Cruz, sc-22538) were diluted to 1:50 and 1:100, respectively. Sections were deparaffinized and rehydrated, then washed in dH₂O and treated with H₂O₂ (10 min). Antigen retrieval for BMP2 was performed using a decloaking chamber (Biocare Medical, 95°C, 10 min) containing sodium citrate buffer (pH 6.0). Antigen retrieval for HIF1a was performed in an oven (37 deg, 15 min) using 5% pepsin. After antigen retrieval,

specimens were cooled and washed in PBS. Sections were blocked with 10% of the appropriate serum (room temperature, 1 hr) and then incubated overnight (4°C) with the optimized dilution of primary antibody. The next day, slides were washed, incubated with a 1:400 dilution of streptavidin (Sigma), washed, incubated with 1:400 of the appropriate secondary antibody, washed, then visualized using 3,3'-diaminobenzidine (DAB) for a predetermined time. Slides were dehydrated, counterstained lightly with Mayer's hematoxylin, coverslipped, and examined under light field microscopy. PCNA immunohistochemistry was done according to the manufacturer's protocol (Invitrogen). The antibody was visualized with DAB after exposure for 5 minutes. All results were assessed qualitatively.

Vascular Perfusion

Vascular perfusion was performed on a subset of rats 3 days after loading (14 rats total; n=4-5/loading group) as previously described [32]. Comparisons were made between WBF loading and rest-inserted LBF loading. Three days after loading the rats were anesthetized by intraperitoneal injection of ketamine (87 mg/kg) and xylazine (13 mg/kg). Heparin (10 mL, 100 USP units/mL) was injected (18-gauge catheter in left ventricle) to inhibit clotting. The vasculature was irrigated with 100 mL saline and 60 mL of a silicone rubber solution was injected (MICROFIL[®], MV-122; Flow Tech). Following injection, the specimens were stored at 4°C overnight to allow the rubber to cure. The forelimbs were dissected and placed in 10% formalin overnight. MicroCT (uCT 40, Scanco Medical, 45 kV, 200 ms, 16 µm resolution) was used to image 1.6 mm of the central ulna starting 1 mm distal to the midpoint and ending 0.4 mm proximal to the midpoint. The forelimbs were then decalcified in 14% EDTA for 3 weeks. Thin (5 µm) sections were cut 1 mm distal to the midpoint from paraffin embedded blocks and mounted on glass slides. Slides were stained with hematoxylin and eosin (H&E) before being analyzed for vessel number and area (Bioquant). A number of rats (n=4 for WBF loading and n=1 for LBF loading) were excluded from analysis due to poor perfusion.

Statistics

Two-way analysis of variance (ANOVA) was used to compare across loading groups and timepoints with differences between individual loading groups and timepoints assessed using Fisher's protected least significant difference test. Differences between right and left ulnae were assessed using paired t-tests. Significance was defined at $p < 0.05$.

Results

A single bout of loading stimulates increased lamellar bone formation without damage

We subjected forelimbs to a single bout of LBF loading using either rest-inserted (0.5 s triangle load-unload followed by 9.5 s rest) or haversine (2 Hz) waveform, both with a peak force 15 N for 100 cycles. Results indicate that both loading protocols were non-damaging and stimulated lamellar bone formation. Three-point bending tests demonstrated no significant differences in structural properties between loaded and control ulnae (Table 2). Moreover, there were no obvious difference in basic fuchsin staining between loaded and control ulnae. In contrast, ulnae loaded by a damaging fatigue protocol (WBF loading) had diffuse staining, indicating extensive damage (Figure 1). Dynamic histomorphometry confirmed that ten days after WBF loading there was abundant new woven bone at the ulnar mid-diaphysis (Figure 1). In sharp contrast, 10 days after LBF loading there was no woven bone observed on loaded or control specimens. Moreover, lamellar bone formation was increased by a similar amount on ulnae loaded with either LBF loading protocol compared to control (Figure 1). Mineralizing surface (MS/BS) was increased 50% in loaded ulnae vs. non-loaded controls, while BFR/BS and MAR were increased more than 80 and 20%,

respectively ($p < 0.05$) (Table 2). In summary, both LBF loading protocols satisfied all of the criteria for a non-damaging, single-bout induction of lamellar bone formation. In addition, comparison of non-loaded controls (left ulnae) to “normal” ulnae (pooled right and left ulnae) demonstrated no significant difference in bone formation parameters.

Cell proliferation is upregulated early after WBF loading, but not for LBF loading

The cell proliferation marker *Hist4* was upregulated 5- and 13-fold on days 1 and 3, respectively, for WBF loading but was unchanged for LBF loading (regardless of loading protocol) compared to control (Table 3, Figure 2). WBF loading also strongly upregulated the cell cycle gene *Ccnd1* (5- and 17-fold on days 1 and 3, respectively), whereas LBF loading led to only a modest increase on day 3 (2-fold). Histologically, there was an increase in cellularity at the periosteum seen on H&E stained sections on day 1 after WBF loading, but no qualitative differences between controls and ulna loaded with the LBF loading protocol (Figure 3). Consistent with qRT-PCR, PCNA staining was absent on sections from control and ulnae loaded with the LBF loading protocol on days 1 and 3. In contrast to LBF and consistent with the above gene expression results, there was PCNA staining after WBF loading on the medial and lateral sides of the ulna on days 1 and 3 (not shown).

Early angiogenesis distinguishes WBF from LBF loading

Expression of *Vegf* and *Hif1a* were upregulated 1.6-fold one hour after WBF loading (Table 3, Figure 2), indicating an early angiogenic stimulus. Expression of *Hif1a* increased to 9- and 15-fold on days 1 and 3 after WBF loading, whereas LBF loading for rest-inserted or haversine waveforms led to only a small increase on day 3 (less than 1.5-fold). WBF loading upregulated *Vegf* and *Pecam* expression on days 1 and 3 (between 3- and 5-fold) while LBF loading only increased expression on day 3 (less than 1.6-fold). Consistent with qRT-PCR, histological staining of HIF1a was increased in ulnar sections after WBF loading, but not after LBF loading on days 1 (Figure 3) and 3 (not shown). Bone morphogenetic protein 2 (*Bmp2*), known as an osteogenic-angiogenic coupling factor [33], was upregulated at all timepoints after WBF loading (6-fold on days 1 and 3). LBF loading using a rest-inserted waveform upregulated *Bmp2* at days 0 and 3, while LBF loading with a haversine waveform was upregulated only at day 3 (2-fold). Immunohistochemistry for BMP2 on day 1 showed protein localized to some periosteal lining cells as well as the interior lining (intima) of most blood vessels (Figure 3). Histological results for BMP2 on day 3 (not shown) were similar to day 1.

WBF loading led to increased vascularity on day 3 as assessed by microCT and histological analysis of perfused ulnae (Figure 4). Vessel number, area and volume were all increased significantly when comparing WBF to LBF loading, with no differences between LBF loading and control.

Genes associated with osteogenesis are highly upregulated on days 1 and 3 after WBF loading but only moderately upregulated on day 3 after LBF loading

Expression of *Osx* was reduced 2-fold one hour after WBF loading, but increased 3.8- and 24-fold by days 1 and 3, respectively (Figure 2, Table 3). WBF loading also upregulated expression of *Runx2* (3- and 8-fold on days 1 and 3, respectively), *Bsp* (14- and 89-fold on days 1 and 3, respectively) and *Bglap* (4-fold on day 3). The fold changes for osteogenic gene expression markers were significantly higher as a result of WBF loading compared to LBF loading. Nevertheless, gene expression was significantly upregulated when comparing LBF loading to control on day 3 (between 1.4- and 2.5-fold increase) for both rest-inserted and haversine waveforms. With regard to bone development, the hedgehog pathway was activated as evidenced by increased *Ptch* expression on day 3 for both WBF and LBF loading (7- and 1.5-fold, respectively).

Discussion

The objectives of our study were to establish a single-bout loading protocol to stimulate only lamellar bone formation, and then to compare the processes associated with loading-induced lamellar and woven bone formation. We identified two single-bout loading protocols that stimulated a significant increase in measures of periosteal bone formation in the loaded ulna without inducing woven bone formation or structural damage. In support of our hypothesis, there were dramatic differences in gene expression levels between loading protocols that produced woven and lamellar bone. The cellular proliferation marker *Hist4* was upregulated at both days 1 and 3 for woven bone formation consistent with a dramatic increase in sub-periosteal cellularity. In contrast, *Hist4* remained at control levels for lamellar bone and there was no evidence of increased cellularity. In particular, strong upregulation of angiogenic genes *Vegf* and *Hif1a* was seen on all days for woven bone, but only a modest upregulation was noted on day 3 for lamellar bone. In addition, vessel number, area and volume were all significantly increased following WBF loading, but not after LBF loading. Taken together, these results indicate the importance of cellular proliferation and angiogenic support for woven, but not for lamellar bone formation following mechanical loading.

One of the strengths of our study was the synchronization of the timelines for induction of woven and lamellar bone formation. Using mechanical loading to induce lamellar bone formation in the rat skeleton has been documented previously, although most studies have used multi-day loading protocols [6,27,34-35]. For the purposes of our study, we needed a one-day protocol because we wanted to document effects relative to a single time-zero, rather than additive effects of multiple days as has been described previously [22,36]. There is one report describing endocortical lamellar bone formation in the rat tibia after a single bout of loading, although that report also described woven bone periosteally [5]. A recent report using a single-bout protocol in young mice for tibial loading resulted in no apparent woven bone formation [37]. Because our objective was to compare lamellar versus woven bone formation, we needed to ensure that the “lamellar” protocol did not produce any woven bone in the region where tissue was harvested for RNA extraction. Some previous reports of gene expression following mechanical loading have not described the type of bone that was induced [22,36], although based on the loading model used (murine tibial bending) it is likely that the loading response would be a combination of periosteal woven bone and endocortical lamellar bone [38-39]. In this study we were able to use single-day loading protocols to compare and contrast the gene expression and vascular responses related to woven and lamellar bone formation.

We investigated two separate LBF loading protocols and showed that stimulating the forelimb with either a rest-inserted or a haversine waveform results in essentially the same gene expression profile. The biggest differences between the loading protocols were the total loading time and displacement change during loading (17 min., 0.1 mm for rest-inserted vs. 1 min., 0.35 mm for haversine waveforms). These differences did not affect the structural properties of the bone, the amount of damage assessed by basic fuchsin staining or the bone formation parameters. A total displacement of 0.6 mm produced by a 2 Hz haversine waveform has been shown to result in a loss of structural properties and woven bone formation [11]. Our preliminary waveform testing (Table 1) also demonstrated woven bone formation at various combinations of displacement and force, indicating that woven bone formation is not simply the result of high displacement or high force. Cycle number, total displacement and peak force are all important factors in determining whether woven or lamellar bone is formed. Importantly, both the timing and the amplitude of the gene expression profiles for WBF loading are different from LBF loading (either waveform). Within 24 hours of WBF loading all selected genes were significantly upregulated, whereas only *Bmp2* was upregulated for LBF loading. By day 3, fold changes in expression increased

up to 45-fold comparing WBF to LBF loading. In contrast, the profiles for the two LBF loading waveforms (rest-inserted and haversine) are not significantly different.

Relatively little is known about the role of cellular proliferation in loading-induced bone formation. In the current study we observed upregulation in markers of cellular proliferation as early as 1 day after WBF loading, prior to woven bone formation. By 3 days after WBF loading there was a 17-fold upregulation of the cell cycle gene *Ccnd1* and a 13-fold upregulation of the proliferation marker *Hist4*. These results are consistent with our previous stress fracture study [12] and with studies of fracture healing [40-41] and highlight the role of cellular proliferation in bone injury responses. In contrast, markers of cellular proliferation were not significantly upregulated 1 day after LBF loading, prior to lamellar bone formation. On day 3 after LBF loading, *Ccnd1* (but not *Hist4*) was only modestly upregulated (1.7 fold). Similar to these results, a previous study reported no increase in proliferating osteoblasts in association with lamellar bone formation in the first two days after tibial bending, but a modest increase starting on day 3 [21]. Thus, available evidence indicates that lamellar bone formation, in contrast to woven bone formation, does not involve an immediate or strong proliferative response.

The role of angiogenesis has been well documented in skeletal development [42-44], fracture repair [19,45-46], and distraction osteogenesis (DO) [3,16,47], scenarios where bone formation occurs rapidly. For example, DO has been shown to be dependent on both VEGFR1 and VEGFR2 signaling [48]. In the current study, we showed an early angiogenic response following WBF loading, including upregulation of *Vegf* and *Hif1a* within 1 hour, consistent with previous reports of early *Vegf* expression [12-13]. By day 3, there was a significant increase in vascularity in the region where woven bone was starting to form, consistent with a previous study [32]. Because the healing response in this model is characterized by non-endochondral bone formation [28], it differs from fracture healing and DO, which include both non-endochondral and endochondral bone formation [20,49]. Nonetheless, all of these healing scenarios involve a strong angiogenic response. In sharp contrast, we observed only a modest change (< 2-fold) in *Vegfa* and *Hif1a* expression on day 3 following LBF loading. Furthermore, LBF loading did not lead to a detectable increase in vascularity on day 3 compared to control. Our results are not consistent with a previous report that lamellar bone formation induced by 2 weeks of treadmill running in young rats was associated with increased *Vegf* expression and vascularity [50]. In that study young, growing rats were used and the focus was on bone formation in the trabecular compartment, which differs from the periosteal bone formation we were investigating in adult rats. The results of our study strongly suggest that periosteal lamellar bone formation in adult rats does not require an increase in vascularity, whereas periosteal woven bone formation does.

We observed increased expression of *Bmp2* in response to both WBF and LBF loading. The early upregulation seen after WBF loading is consistent with previous reports from stress fracture [12-13] and fracture [51] studies, and suggests that BMP2 may play a critical role in stress fracture healing, as it does for fracture healing [52]. BMP2 has been shown to stimulate angiogenesis by upregulation and secretion of *Vegf* by osteoblasts [53], which suggests that BMP2 acts as an osteogenic-angiogenic coupling factor. This is consistent with our results after WBF loading, where both *Bmp2* and *Vegf* were upregulated along a similar timecourse prior to woven bone formation. However, osteogenesis and angiogenesis did not appear to be coupled in lamellar bone formation in our study, as rest-inserted LBF loading led to an early increase in *Bmp2* without a coordinated increase in *Vegf* expression. Rather, the early upregulation in response to LBF loading suggests that BMP2 is mechanoresponsive, which is consistent with previous reports in osteoblasts [54] and endothelial cells [55].

Upregulation of osteogenic genes after mechanical loading has been reported previously. For example, 4 to 8 days of murine tibial bending induced increases of 2- to 4-fold in expression of bone sialoprotein and osteocalcin [36]. The authors noted that their results likely reflected contributions from both lamellar and woven bone. In our study, examination of woven and lamellar bone separately demonstrated that woven bone formation was associated with earlier and significantly greater upregulation of osteogenic genes. On day 1, *Bsp*, *Runx2* and *Osx* were upregulated by 3- to 14-fold with woven bone formation but were not significantly upregulated with lamellar bone formation. By day 3, these genes were upregulated 8- to 89-fold for woven bone compared to approximately 2-fold for lamellar bone. The terminal differentiation marker osteocalcin was not significantly upregulated until day 3 in our study, regardless of the type of loading. Similarly, Kesavan et al. reported no upregulation of osteocalcin after 4 days of loading but an increase by day 8. For woven bone, the dramatic upregulation in expression of genes related to osteoblast differentiation along with the increase in cellular proliferation and expansion of the periosteum indicates that the number of osteoblasts is being greatly increased, likely from a pool of progenitor cells. By contrast, for lamellar bone, the modest increase in osteoblast differentiation markers with only negligible cellular proliferation and expansion of the periosteum indicates that existing osteoblasts and pre-osteoblasts are sufficient to support lamellar bone formation. Despite these differences in expansion of the osteoblast pool, by day 3 each of the osteogenic genes we analyzed was upregulated for both woven and lamellar bone formation. It is likely that the differences in magnitude of the gene expression responses between woven and lamellar bone formation correlate with the volume of bone formed. This is consistent with our previous study wherein an increasing amount of woven bone was associated with an amplification of osteogenic gene expression [12].

There were several limitations to our study. First, although our data suggest that woven bone is dependent on angiogenesis, we have yet to prove that in the absence of angiogenesis woven bone is inhibited. Future work will address this issue. Second, the number of loading cycles is variable in the WBF loading model ranging from 469 to 23463 (4-195 minutes). This variability in cycle number has been well documented in this loading model [11-12,28,32,56] and was not correlated with any of our experimental outcomes. Third, one consideration in our WBF loading scenario is the formation of lamellar bone that occurs adjacent to woven bone. To minimize the influence of lamellar bone formation in the samples evaluated after WBF loading we confined our gene expression region of interest to 5 mm near the site of the stress fracture, an area shown to produce predominantly woven bone [28]. Moreover, by comparing WBF to LBF loading in which woven bone is absent, any differences in gene expression reflect the woven bone component. On the other hand, comparisons between control and LBF loading describe any events associated only with increased lamellar bone formation. Finally, recent reports have suggested fatigue loading of the ulna induces periosteal bone formation in the contralateral ulna [31,57], which could influence comparisons between right and left ulnae. Similar to a study in mice [58] we found no difference in bone formation parameters between non-loaded left ulnae and “normal” ulnae. This validates our usage of the non-loaded left ulna as an internal control in our model.

In conclusion, lamellar bone formation can be initiated using a single bout of LBF loading in the rat ulna. There were no differences in structural properties, bone formation parameters or gene expression values between LBF protocols which used a rest-inserted or haversine waveform. In contrast, we showed differences in timing and amplitude of gene expression between loading protocols that stimulated woven and lamellar bone formation. WBF loading increased expression of genes related to cell proliferation and angiogenesis while increasing vascularity at day 3. In addition, WBF loading led to high fold-increases in osteogenic gene expression, consistent with the large amount of woven bone formation observed

histologically on day 10. By contrast, LBF loading did not increase cell proliferation or angiogenic gene expression at early timepoints, and did not result in an increase in vascularity. There was a modest upregulation of osteogenic gene expression 3 days after LBF loading, followed by lamellar bone formation seen histologically. In summary, histological evidence indicates that the cellular proliferation and angiogenic responses are different between woven and lamellar bone formation. The expression profiles demonstrated an earlier and more robust gene activation following WBF loading and a later, lesser response following LBF loading.

Acknowledgments

NIH/NIAMS R01AR050211, P30AR057235 (Washington University Center for Musculoskeletal Research); ASBMR.

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Figure 1.

WBF loading (18 N haversine waveform; 1.3 mm total disp.) led to formation of woven bone, while LBF loading (either rest-inserted or haversine waveform; 100 cycles) led to formation of lamellar bone. Basic fuchsin staining on day 0 (immediately after loading) was used to assess damage on bright field [inset] and fluorescent images. Ulnae subjected to WBF loading had high amounts of diffuse ulnar staining and a large crack while both LBF loading and non-loaded control ulnae had no diffuse damage or cracks. Representative histological sections 10 days after loading illustrate extensive woven bone formation after WBF loading. There is increased labeled surface around the periosteum after LBF loading (rest-inserted waveform shown) compared to non-loaded control. Images for haversine LBF loading (not shown) were not different from rest-inserted LBF loading. All sections were taken 1 mm distal to the midpoint (D1). (scale bar 500 micron)

Figure 2.

Gene expression differed in timing and amplitude between WBF and LBF loading, but not between LBF loading protocols using rest-inserted or haversine waveforms. Cell proliferation markers *Hist4* and *Ccnd1* demonstrate a significant increase (loaded/non-loaded) on both days 1 and 3 only in the ulnae stimulated to produce woven bone. There was no increase in *Hist4* after LBF loading at any timepoint. *Bmp2* was significantly upregulated for both LBF and WBF loading at various timepoints. *Hif1a* is upregulated on days 1 and 3 for woven bone, over 10-fold higher than lamellar bone at day 3. Expression of osteogenic markers was increased at days 1 and 3 for woven bone, with a smaller increase at day 3 for lamellar bone. The upregulation in *Bsp* was over 8 and 45 times larger than lamellar bone on days 1 and 3, respectively. (* p<0.05 Loaded vs. non-loaded)

Figure 3.

Histological sections on day 1 demonstrate differences in cell proliferation, expression of BMP2 and HIF-1a proteins. At low magnification, hematoxylin and eosin (H&E) stained sections demonstrate cortical bone cracks following WBF loading (stimulating woven bone). At higher magnification H&E sections show that WBF loading stimulated periosteal expansion and increased cell proliferation (not shown). LBF loading (rest-inserted stimulation of lamellar bone) did not exhibit periosteal expansion compared to control. Immunohistochemistry at day 1 showed increased localized BMP2 and HIF1a protein following stimulation of woven compared to lamellar bone formation. WBF loading increased BMP2 staining in a small number of lining cells (thin arrow), as well as in connective tissue and blood vessels both in the periosteum (block arrow) and muscle (not shown). Both loading protocols and controls had BMP2 staining in neuro-vascular bundles (arrowhead). HIF-1a staining is much more prominent in neutrophils after WBF loading (black arrow), and is present in small amounts after LBF loading. All histological sections are taken 1 mm distal to the midpoint (scale bar 50 μ m; b—original cortical bone; p—periosteum; m—muscle)

Figure 4.

Vascularity increased in response to WBF loading. A) The vascular response was assessed on day 3 using histological sections (stained with H&E) and microCT imaging. Sections for control and LBF loading (rest-inserted waveform) are qualitatively different from WBF loading sections (vessels are black dots). Similarly on microCT the appearance of blood vessels is increased after WBF loading. B) Quantitatively, there is an increase in the total vessel number, area and volume of vessels associated with woven bone formation compared to control. There is no difference in vessel number, area or volume between ulnae loaded with the rest-inserted LBF protocol and controls. (n=4-5/group; scale bar 500 micron)

Table 1

Multiple waveforms were screened for use in a single-bout lamellar loading protocol; the highlighted waveforms were further characterized

	Rest-inserted waveform (0.5 s load-unload followed by 9.5 s rest)				Haversine waveform (2 Hz)			
Number of rats	12	2	6	7	11	7	10	10
Peak loading force (N)	15	15	18	14	15	15	16	18
Loading cycles	100	200	100	230	100	230	230	230
Total disp. (mm)	0.087 ± 0.051	0.173 ± 0.062	0.108 ± .042	0.413 ± 0.015	0.355 ± 0.039	0.399 ± 0.047	0.365 ± 0.028	0.407 ± 0.04
Loading-induced woven bone formation?	No (0/12)	No (0/2)	Yes (2/6)	No (0/7)	No (0/11)	Yes (1/7)	Yes 2/10	Yes (5/10)

Table 2
Mechanical property (day 0) and bone formation (day 10) outcomes following lamellar bone formation (LBF) loading

		Number of rats	Mechanical Properties			Periosteal Bone Formation			
			Ultimate Force (N)	Stiffness (N/mm)	Energy to Fracture (N*mm)	Number of rats	MS/BS (%)	BFR/BS (µm/day)	MAR (µm/day)
Rest-inserted waveform	Non-loaded	12	12.9 ± 1.4	20.9 ± 3.0	22.7 ± 4.1	12	47.3 ± 16.2	0.68 ± 0.32	1.41 ± 0.45
	Loaded	12	12.8 ± 0.9	20.2 ± 1.7	26.4 ± 5.2	12	74.0* ± 11.7	1.30* ± 0.30	1.75* ± 0.20
Haversine waveform	Non-loaded	3	16.9 ± 2.2	28.3 ± 4.9	24.8 ± 2.1	11	50.0 ± 11.2	0.77 ± 0.18	1.56 ± 0.27
	Loaded	3	16.8 ± 0.9	28.0 ± 2.1	22.6 ± 2.6	11	72.2* ± 22.6	1.38* ± 0.52	1.89* ± 0.22
Normal	Non-loaded rat	-	-	-	-	6	45.4 ± 15.4	0.66 ± 0.36	1.39 ± 0.29

Mineralizing surface per bone surface (MS/BS), bone formation rate per bone surface (BFR/BS) and mineral apposition rate (MAR) were measured 1 mm distal to the midpoint of the ulna.

* p<0.05 vs. non-loaded

Table 3

Fold changes in expression (loaded/control) were greater for WBF than for LBF loading

Gene Name	Gene Symbol	WBF loading (woven bone)			LBF loading, rest-inserted waveform (lamellar bone)			LBF loading, haversine waveform (lamellar bone)		
		Day 0 (1 hr)	Day 1	Day 3	Day 0 (1 hr)	Day 1	Day 3	Day 0 (1 hr)	Day 1	Day 3
Histone H4	<i>Hist4</i>	0.87 ± 0.46	5.69 ^a ± 4.15	13.41 ^a ± 4.71	0.98 ± 0.33	1.06 [#] ± 0.65	0.75 [#] ± 0.35	0.99 ± 0.70	0.93 [#] ± 0.61	0.94 [#] ± 0.66
Cyclin D1	<i>Ccnd1</i>	1.37 ± 0.39	4.99 [*] ± 1.51	16.86 ^a ± 5.31	1.42 ± 0.57	1.69 [#] ± 1.28	1.86 ^a ± 0.51	1.32 ± 0.58	1.47 [#] ± 0.52	1.72 ^a ± 0.46
Vascular endothelial growth factor	<i>Vegf</i>	1.69 [*] ± 0.26	3.03 ^a ± 1.24	3.57 ^a ± 0.50	1.08 [#] ± 0.14	1.15 [#] ± 0.22	1.20 ^a ± 0.11	1.07 [#] ± 0.27	1.05 [#] ± 0.35	1.12 [#] ± 0.14
Hypoxia-inducible factor 1, alpha subunit	<i>Hif1a</i>	1.60 [*] ± 0.40	9.19 ^a ± 3.14	15.18 ^a ± 3.83	1.23 ± 0.30	1.26 [#] ± 0.44	1.44 ^a ± 0.34	1.11 [#] ± 0.35	1.40 [#] ± 0.59	1.25 ^a ± 0.24
Platelet/endothelial cell adhesion molecule 1	<i>Pecam</i>	1.15 ± 0.29	2.54 ^a ± 0.54	4.73 ^a ± 0.88	1.14 ± 0.29	1.15 [#] ± 0.36	1.58 ^a ± 0.33	1.09 ± 0.32	1.21 [#] ± 0.45	1.21 ^a ± 0.06
Bone morphogenetic protein 2	<i>Bmp2</i>	2.53 [*] ± 0.46	6.50 ^a ± 4.03	6.98 ^a ± 2.49	1.94 [*] ± 0.51	1.70 [#] ± 0.84	1.90 ^a ± 0.44	1.42 ± 0.67	1.61 [#] ± 1.01	1.85 ^a ± 0.60
Bone sialoprotein	<i>Bsp</i>	1.16 ± 0.25	13.57 [*] ± 4.92	88.98 ^a ± 28.84	1.10 ± 0.38	1.57 [#] ± 0.74	1.93 ^a ± 0.31	1.00 ± 0.42	1.51 [#] ± 0.86	1.72 ^a ± 0.40
Runt related transcription factor 2	<i>Runx2</i>	1.03 ± 0.31	2.55 [*] ± 0.78	8.18 ^a ± 2.57	1.05 ± 0.42	1.31 [#] ± 0.51	1.61 ^a ± 0.35	1.00 ± 0.38	1.28 [#] ± 0.51	1.42 ^a ± 0.28
Osteocalcin	<i>Bglap</i>	0.99 ± 0.31	0.39 [*] ± 0.10	3.85 ^a ± 1.09	1.03 ± 0.42	1.43 [#] ± 0.67	2.24 ^a ± 0.64	0.96 ± 0.33	1.19 [#] ± 0.55	1.85 ^a ± 0.59
Osterix	<i>Osx</i>	0.5 [*] ± 0.17	3.81 [*] ± 2.03	24.01 ^a ± 9.53	1.00 [#] ± 0.35	1.61 [#] ± 0.76	2.14 ^a ± 0.45	0.93 ± 0.44	1.43 [#] ± 0.68	1.92 ^a ± 0.51
Patched homolog 1	<i>Ptch</i>	0.86 ± 0.33	1.97 [*] ± 0.97	6.65 ^a ± 2.92	0.94 ± 0.32	1.46 ± 0.69	1.56 ^a ± 0.30	1.09 ± 0.43	1.30 ± 0.31	1.37 ^a ± 0.13

* p<0.05 vs. control

^a p<0.05 vs. Day 0

^b p<0.05 vs. Day 1

[#] p<0.05 vs. WBF loading