



Metabolic Endotoxemia and Inhibition of Direct Bone Regeneration: A Pilot Study

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Authors' contributions

This work was carried out in collaboration between all authors. All authors contributed both intellectually and physically to this report. In addition, all the above authors had editorial rights over the final draft. We hereby submit the following: 1) all co-authors approved of this submission, 2) all authors made appropriate contributions, 3) the authors ensure the integrity of the work and 4) none of the above has appeared in any form other than one abstract.

Research Article

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ABSTRACT

Aims: Clinical and pre-clinical studies have demonstrated that alcohol abuse, aging, diabetes and rheumatoid arthritis are associated with increased risk of fractures compounded with impaired fracture repair. We note that these and other pathologies are characterized by chronic inflammation (CI) as a risk factor. How these CI pathologies inhibit bone repair is unclear, but one candidate mediator is endotoxin (lipopolysaccharide/LPS). LPS promotes inflammation and is present in increased serum concentrations in some inflammatory conditions. The distraction osteogenesis (DO) model developed in this laboratory provides the opportunity to isolate and study the effects of CI on direct bone formation during bone regeneration. The aim of this study was to determine whether endotoxin at concentrations that mimic levels reported

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in chronic inflammatory conditions would impair bone formation in a mouse model of DO.

Study Design: Mouse bone repair study.

Place and Duration of Study: Arkansas Children's Hospital Research Institute, Little Rock, Arkansas, April to June 2009.

Methodology: LPS or vehicle (PBS) was chronically administered to 11-week old mice via alzet pump. Mice underwent the DO protocol concurrently with LPS administration. Radiographic and histologic quantitation was performed on the DO gap to determine the amount of new bone formed.

Results: Radiographic ($51.9 \pm 7\%$ vehicle vs $21.0 \pm 7.3\%$ LPS; $P < .01$) and histologic ($68.1 \pm 8.5\%$ vehicle vs $33.6 \pm 10.3\%$ LPS; $P < .02$) results indicate that bone formation during DO was significantly decreased in LPS treated versus vehicle treated mice.

Conclusion: The magnitude of the osteoinhibitory effects of systemic LPS in this mouse model of CI was equivalent to two months of ethanol treatment, 24 months of aging, or two months of Type 1 diabetes. These results support the hypothesis that LPS exposure could be responsible for the decreased bone formation observed in chronic inflammatory conditions.

Keywords: Endotoxemia; LPS; mouse; distraction osteogenesis.

1. INTRODUCTION

Clinical and pre-clinical studies have demonstrated that alcohol abuse, aging, diabetes, and rheumatoid arthritis are associated with increased risk of fractures compounded with impaired fracture repair (Gandhi et al., 2006; Hardy and Cooper, 2009; Purohit, 1997; Wahl et al., 2010). We note that these and other pathologies (i.e. cancer, obesity) are characterized by chronic inflammation (CI) as a risk factor (Calder et al., 2011; Dunn et al., 2012; Medzhitov, 2010; Nathan and Ding, 2010). Bone is a biologically privileged tissue that has the capacity to completely regenerate. The distraction osteogenesis (DO) model developed in this lab provides the opportunity to isolate and study the effects of CI on direct bone formation during bone regeneration (Marsell and Einhorn, 2010; Wahl et al., 2010).

Published studies from this laboratory and others have demonstrated that the tumor necrosis factor alpha/tumor necrosis factor alpha receptor 1 (TNF/TNFR1) axis is a primary osteoinhibitive mediator of the deficits in direct bone repair in rodent models of chronic ethanol exposure (EtOH), aging and diabetes (Assuma et al., 1998; Barbour et al., 2010; Cani et al., 2007; Perrien et al., 2003; Thraillkill et al., 2005; Wahl et al., 2007, 2010). How these CI pathologies induce TNF is unclear. One hypothesis is that moderately increased levels of endotoxin (lipopolysaccharide/LPS) cause chronic low level inflammation and increased levels of TNF which in turn inhibit new bone formation. Endotoxins are LPS components of the outer cell wall of most gram negative bacteria found both orally and in the gut and high levels are seen in CI and EtOH delivery in human and rodent populations (Enomoto et al., 2001a, 2001b; Priø et al., 2002; Tipoe et al., 2008). We note that the marrow and vasculature are intimately involved in bone repair during both fracture healing and DO. Therefore, blood-born LPS have access to both these processes.

A model has been developed to study the effects of low grade CI on mice (Cani et al., 2007). The authors demonstrated that chronic administration of LPS mimicked the effects of high fat diets on insulin resistance, adipose tissue weight gain, inflammatory tone, and that the

serum levels of LPS increased 2 to 3 fold, a threshold which the authors defined as metabolic endotoxemia. Notably these levels are 10 to 50 times lower than those obtained during septic shock. Further, LPS administration was associated with increased serum TNF levels in several organ systems (Cani et al., 2007).

Therefore, in a novel combination of the metabolic endotoxemia protocol and the mouse DO model, we tested the effects of LPS on direct bone repair. As hypothesized, chronic LPS administration (during bone repair) inhibited new bone formation by >50%, which has been found to be equivalent to two months of EtOH administration, 24 months of aging, or six weeks of type 1 diabetes (Thraill et al., 2005; Wahl et al., 2007, 2010).

2. MATERIALS AND METHODS

2.1 Animals

Virus-free adult male C57BL/6 (B6, Jax #000664) mice were purchased from Jackson Industries (Bar Harbor, ME). They were housed in individual cages in temperature (22°C) and humidity (50%) controlled rooms having a 12 h light/12 h dark cycle. All mice were handled by animal care personnel for 5 to 7 days prior to surgery. In all studies, the mice were assigned to respective experimental groups with mean body weights equal to that of the control group ($\pm 4g$) for the study. All research protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arkansas for Medical Sciences.

2.2 Study Design

Since we hypothesized that EtOH induced serum LPS levels could contribute to the inhibition of direct bone formation, we designed this study as a test of the hypothesis that continuous administration of LPS during the distraction protocol would result in osteoinhibition in B6 mice. Twenty 11-week-old male C6 mice underwent placement of an external fixator and osteotomy to the left tibia. At the time of surgery, an alzet pump (model 1002) was inserted subcutaneously between the scapulae on the back of each mouse. The average infusion rate was 0.25 μ l/hour. The alzet pumps were filled with either a low/moderate dose of LPS (600 μ g/kg/day; n=10) or vehicle (saline; n=10) (Cani et al., 2007). The dose was chosen based on the model originated in the Cani paper to reproduce the increase in TNF to levels we have determined to be osteoinhibitive. We note that this dose is 33-fold less than the septic models and ten-fold less than the LPS/fracture paper (Cani et al., 2007; Reikerås et al., 2005). Distraction began three days after surgery (three day latency) at a rate of 0.075 mm b.i.d and continued for 11 days. At sacrifice the distracted and contra lateral tibiae were harvested and trunk blood was collected for serum TNF levels. All mice survived to harvest.

2.3 Distraction Protocol

Briefly as previously published, following acclimation and under Nembutal anesthesia, each mouse underwent placement of an external fixator and osteotomy to the left tibia (Wahl et al., 2010). Four 27-gauge, 1.25-in needles were manually drilled through the tibia (two proximally, two distally). The titanium external fixator was then secured to the pins. A small incision was made in the skin distal to the tibia crest and the soft tissue was carefully retracted to visualize the bone. A single hole was manually drilled through both cortices of

the mid-diaphysis, and surgical scissors were used to fracture the cortex on either side of the hole. The fibula was fractured by direct lateral pressure. The periosteum and dermal tissues were closed with a single suture. Finally, buprenex (1.0 mg/kg) was given by intramuscular injection post surgery for analgesia.

2.4 Radiographic and Histologic Analysis

After 48 hours of fixation in 10% neutral buffered formalin, the left tibiae were removed from the fixators for high-resolution single beam radiography and subsequent histological processing (Skinner and Fromowitz, 1989). For initial radiography, a Xerox Micro50 closed system radiography unit (Xerox, Pasadena, CA, USA) was used at 40 kilovolts (3 mA) for 20 seconds using Kodak X-OMAT film. For quantification, the radiographs were video recorded under low power (2X objective) microscopic magnification, and the area of mineralized new bone in the distraction gaps were evaluated by NIH Image Analysis 1.62 software/Image J software 1.30 (rsb.info.nih.gov/ij/). The measured distraction gap was outlined from the outside corners of the two proximal and the two distal cortices forming a quadrilateral region of interest. The mineralized new bone area in the gap was determined by visually outlining the regions with radiodensity equivalent to or greater than an adjacent non-bone (background) area (excluding bone chips). The percentage of new mineralized bone area within the distraction gap (percent new bone) was calculated by dividing mineralized bone area by total gap area. Therefore, the percent new bone as measured by the radiograph analysis is an estimate of new "mineralized" bone in the entire gap.

After radiography, the distracted tibiae were decalcified in 5% formic acid, dehydrated, and embedded in paraffin (Skinner et al., 1997). Experience in our laboratory has demonstrated that this approach achieves good morphology in murine orthopedic tissues and does not appear to significantly impair immunological detection of many epitopes (Perrien et al., 2003). Five to seven micron longitudinal sections were cut on a microtome (Leitz 1512, Wetzlar, Germany) for hematoxylin and eosin staining (H&E). Sections were selected to represent a central or near central gap location. A quadrilateral region of interest was outlined and recorded as follows. Both the proximal and distal endocortical (measured from the inside corners of the cortices) and the intracortical (cortical wall included) new bone matrix was outlined together from the outside edges of the cortices, and the area was recorded as new gap bone formation. The percentage of new gap bone area within the DO gap (percent new bone) was calculated as above. The percent new bone as measured by the histological analysis is an estimate of new gap bone formation which would include non-mineralized osteoid columns, embedded new sinusoids and maturing mineralized bone columns. To be included in both radiographic and histologic analyses, the DO samples had to 1) be well aligned, 2) have no broken pin sites, 3) have few bone chips within the DO gap, 4) have an intact ankle, and 5) have had no significant weight loss or health problems during the distraction period.

2.5 Serum mouse TNF Bead Array

Serum samples were run on a Luminex® 100™ Bioanalyzer in the Pediatric Endocrinology Core Facility, using the Linco mouse adipokine kits (MPXMCYTO-70K-1).

2.6 Serum Endotoxin concentrations

The serum samples were sent to Lonza Corp for endotoxin testing using the KQCL test.

2.7 Statistics

Differences between group means were determined by the Student's t test. All data are reported as mean \pm standard error of the mean (SEM). Differences were considered significant when $P < .05$.

3. RESULTS

We designed this study as a test of the hypothesis that continuous administration of LPS during the distraction protocol would result in osteoinhibition in normal (B6) mice. Comparison of the distracted tibial radiographs demonstrated a significant decrease in LPS treated ($21.0 \pm 7.3\%$) versus controls ($51.9 \pm 7.0\%$; $P < .01$) (Fig. 1). This suggests that LPS treatment reduced the mineralization of newly formed bone in the DO gaps. Further, analysis of the histological sections supported the radiographic findings by revealing a significant decrease in cellular new bone formation in the LPS treated mice ($33.6 \pm 10.3\%$) compared to the vehicle treated mice ($68.1 \pm 8.5\%$, $P < .02$) (Fig. 1). Taken together with the radiographic analysis, this result suggests that cellular osteogenesis and angiogenesis are equivalently inhibited by LPS and account for the reduced later stage mineralization results. Representative radiographs and H&E stained histological sections of distracted tibial DO gaps from LPS and vehicle treated mice are shown in Fig. 2. Surprisingly, the serum TNF levels at harvest were not significantly higher in the LPS group (7.0 ± 1.4 pg/ml) vs. the controls (6.7 ± 1.6 pg/ml). The serum endotoxin levels in the LPS treated mice were 12.3 ± 0.7 as compared to 6.8 ± 0.9 EU/ml in the vehicle controls. One animal was excluded from the analyses.

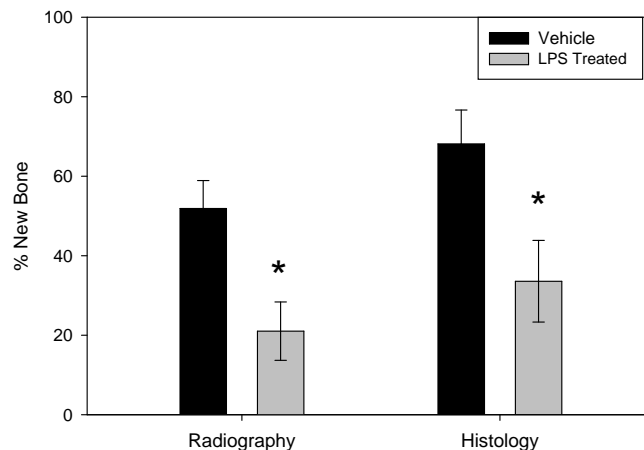


Fig. 1. LPS and bone repair: Comparison of the distracted tibial radiographs demonstrated a significant decrease in LPS treated ($21.0 \pm 7.3\%$) versus controls ($51.9 \pm 7.0\%$; $P < .01$). This decrease suggests that LPS treatment reduced the mineralization of newly formed bone in the DO gaps. Analysis of the histological sections supported the radiographic findings by revealing a significant decrease in cellular new bone formation in the LPS-treated mice ($33.6 \pm 10.3\%$) compared to the vehicle treated mice ($68.1 \pm 8.5\%$, $P < .02$).

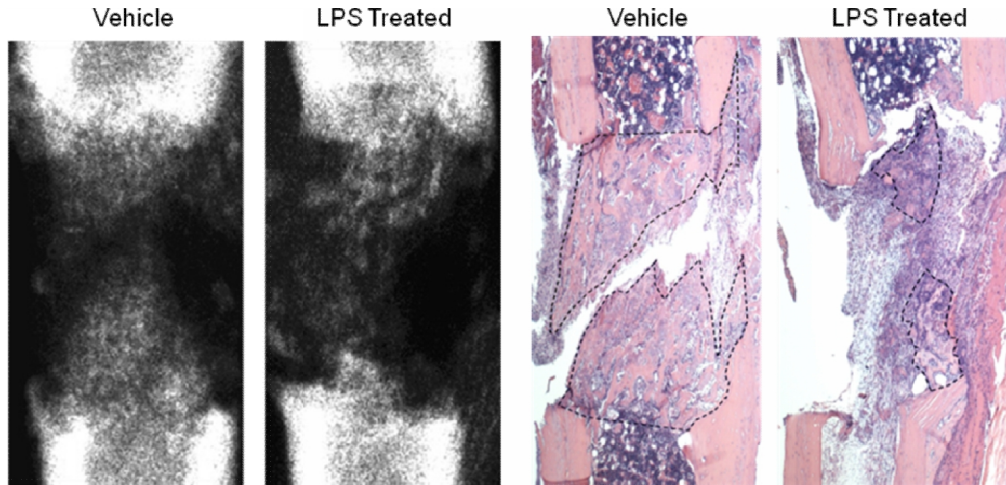


Fig. 2. Left panel - Representative radiographs of distracted tibial DO gaps from LPS- and vehicle-treated mice are shown. Right panel - Representative H&E stained histological sections of distracted tibial DO gaps from LPS- and vehicle-treated mice. The new bone (osteoid/sinusoid) formation is shown outlined by dashed lines.

4. DISCUSSION

It has been suggested that “no single phenomenon contributes more to the medical burden in industrialized societies than non-resolving CI” (Nathan and Ding, 2010). For example, CI contributes significantly to pathogenesis of cancer, aging (“inflammaging”), insulin resistance, atherosclerosis, obesity, pulmonary diseases, asthma, bowel disease, neurodegenerative disease, multiple sclerosis, and rheumatoid arthritis (Medzhitov, 2010; Nathan and Ding, 2010). The goals of this project were to determine the effects of a model of CI on bone formation during DO to begin to determine the possible mechanistic steps between CI, induction of TNF and osteoinhibition.

Published studies from our laboratory and others have demonstrated that TNF is a primary osteoinhibitory cytokine mediating deficits in direct bone repair/regeneration in rodent models of chronic ethanol exposure, aging, and diabetes (Assuma et al., 1998; Barbour et al., 2010; Cani et al., 2007; Perrien et al., 2003; Thrailkill et al., 2005, Wahl et al., 2007, 2010). Based on the results of the studies above, we hypothesized that LPS could be a primary trigger of CI and an upstream inducer of TNF and therefore indirectly contribute to bone repair pathology through the following hypothetical pathway (LPS-TLR4-TNF-TNFR1-NF B-p53-p21-osteoinhibition). Previous studies in liver models have attempted to block the effects of excess LPS in vivo by administration of antibiotics; however, we note that despite some positive effects of these treatments the serum levels of TNF remained above those we have demonstrated as being inhibitory to bone repair (Enomoto et al., 1999). In addition, the ethanol-associated increase in gut permeability was unchanged by antibiotic treatment (Enomoto et al., 2001a, 2001b). Therefore, we have utilized the published mouse metabolic endotoxemia protocol to test for the effects of LPS on the mouse DO model (Cani et al., 2007).

As hypothesized, comparison of the distracted tibial radiographs demonstrated a significant decrease in LPS treated ($21.0 \pm 7.3\%$) versus controls ($51.9 \pm 7.0\%$; $p < 0.01$). This decrease

suggests that LPS treatment reduced the mineralization of newly formed bone in the DO gaps. Analysis of the histological sections supported the radiographic findings by revealing a significant decrease in cellular new bone formation in the LPS-treated mice ($33.6 \pm 10.3\%$) compared to the vehicle treated mice ($68.1 \pm 8.5\%$, $p < 0.02$). Comparatively, chronic LPS administration (during bone repair) inhibited new bone formation at a percent equivalent to two months of chronic ethanol administration, 24 months of aging, or six weeks of type 1 diabetes (Thraill et al., 2005; Wahl et al., 2007, 2010). To date, a few recent papers have reported the effects of systemic LPS on bone, one of which implicates TNFR1 (Hardy et al., 2009; Ochi et al., 2010). One report demonstrates that the LPS-induced inhibition of bone formation after tooth extraction is TNF dependent (Tomomatsu et al., 2009). The tooth extraction model is a single LPS injection and a onetime extraction and therefore may not be comparable to the study here; however, the connection between LPS and TNF deserves note. Finally, in agreement with the results reported here, a paper demonstrated the osteoinhibitive effects of LPS on fracture healing (Reikerås et al., 2005). We note that the cellular organization and isolation of osteoblastogenesis offered by the DO process makes it a suitable model for the studies of LPS's effects on direct bone formation. Fracture healing is a more mixed repair model, which contains both endochondral (indirect) and direct bone formation. When comparing DO to fracture healing, we note that direct bone formation in both processes is, to date, identical at the cellular and molecular levels. We hypothesize that deficits in fracture healing involving direct bone formation in the clinic can be modeled by DO in the laboratory.

Taken together, the published results support our hypothesis that LPS can induce osteoinhibition. We note that the serum TNF levels at harvest were not significantly higher in the LPS group vs. the controls. There may be a number of reasons for this. For example, tolerance to LPS has been shown in many other models, where prior exposure of cells to low doses of LPS reduces the TNF response to subsequent low doses of LPS. In our model, LPS was administered chronically throughout the experimental protocol, but TNF was measured only at the harvest endpoint day 14, when cells could have become desensitized to the LPS. In fact, in a mouse model of tooth extraction, the authors demonstrated that LPS induced osteoinhibition was mediated by TNF and the inhibition was significant even at 21 days post extraction. However, the serum TNF levels peaked at day 3 and were not detected at day 21 (Tomomatsu et al., 2009). Further, in a recent paper TNF was required for induction but not maintenance of bone marrow edema in a mouse tail vertebrae compression model (Papuga et al., 2011). In addition, the initial excess of TNF could induce signaling through the high mobility group box protein 1 (HMGBP-1)/receptor advanced glycation end products (RAGE) axis, which is known to prolong inflammation (Bianchi and Manfredi, 2009; Lamore et al., 2010; Maroso et al., 2010). HMGBP-1 has recently been shown to bind to and transfer LPS to CD14, consequently increasing induction of TNF (Youn et al., 2011). In this context we note that, to date, study of LPS/TNF induction of HMGBP-1 and the resultant effects on bone repair would be a novel direction. We also note the possibility of TNF-independent LPS effects on bone repair (other osteoinhibitive factors). Further systemic TNF levels may not reflect local gap TNF levels. Taken together, the LPS may be inducing osteoinhibitive levels of TNF over the early part of the protocol which could delay the repair process and/or trigger factors that continue to mediate the osteoinhibition.

Future studies employing chronic LPS administration will be needed to measure serum TNF at several time points during the experiment to determine whether tolerance to LPS is occurring. Another possible reason for the lack of change in TNF in the LPS model is that the chronic low dose of LPS used in our study might not have been sufficient to induce systemic TNF, but was able to induce local production of TNF (e.g., in the distraction gap).

Considering LPS levels rise with blood ethanol levels, aging, diabetes, and high fat diets in mice, rats, and humans; these results support the working hypothesis that LPS levels may contribute to the consequent osteoinhibition in several pathologies. Further, we believe this model may mimic the human clinical condition with respect to CI. It has been suggested that the LPS model used here is a “sterile” inflammation model and therefore not applicable to the human situation. We disagree. In the study that resulted in the development of the metabolic endotoxemia model we employed here, the authors demonstrated that LPS was the microbial trigger of low grade inflammation, insulin resistance and atherosclerosis due to high fat diets (Cani et al., 2007). They also demonstrated that delivery of LPS alone reproduced all the conditions of the high fat diets. Further it has been well established that LPS is the most active, longest lived, and most predictive microbial component in human diseases such as type 2 diabetes, obesity and atherosclerosis (Cani and Delzenne, 2009).

5. CONCLUSION

In conclusion, we believe the combination of the endotoxemia model and the bone regeneration model can provide insights into pathologies associated with CI that are characterized by increased risk of fractures compounded with impaired fracture repair.

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ETHICAL APPROVAL

All authors hereby declare that “principles of laboratory animal care” (NIH publication no. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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