Published in final edited form as:

Toxicol Appl Pharmacol. 2007 May 1; 220(3): 302–310.

Chronic Ethanol Exposure Inhibits Distraction Osteogenesis in a Mouse Model:

Role of the TNF Signaling Axis

Elizabeth C. Wahl, James Aronson, Lichu Liu, Zhendong Liu, Daniel S. Perrien, Robert A. Skinner, Thomas M. Badger, Martin JJ Ronis, and Charles K. Lumpkin Jr

Departments of Pediatrics (JA, CKL), Orthopaedics (JA, RAS), Physiology and Biophysics (TMB), Pharmacology and Toxicology (MJJR), College of Medicine University of Arkansas for Medical Sciences, Little Rock, AR. Laboratory for Limb Regeneration Research (ECW, DSP, JA, LL, ZL, CKL), Arkansas Children's Hospital Research Institute, Little Rock, AR. Arkansas Children's Nutrition Center (TMB, MJJR), Little Rock, AR.

Abstract

Tumor necrosis factor- α (TNF- α) is an inflammatory cytokine that modulates osteoblastogenesis. In addition, the demonstrated inhibitory effects of chronic ethanol exposure on direct bone formation in rats is hypothetically mediated by TNF- α signaling. The effects in mice are unreported. Therefore, we hypothesized that in mice 1) administration of a soluble TNF receptor 1 derivative (sTNFR1) would protect direct bone formation during chronic ethanol exposure, and 2) administration of recombinant mouse TNF- α (rmTNF- α) to ethanol naïve mice would inhibit direct bone formation. We utilized a unique model of limb lengthening (distraction osteogenesis, DO) combined with liquid diets to measure chronic ethanol's effects on direct bone formation. Chronic ethanol exposure resulted in increased marrow TNF, IL-1, and CYP 2E1 RNA levels in ethanol treated vs control mice, while no significant weight differences were noted. Systemic administration of sTNFR1 during DO (8.0 mg/kg/2 days) to chronic ethanol exposed mice resulted in enhanced direct bone formation as measured radiologically and histologically. Systemic rmTNF- α (10 μ g/kg/day) administration decreased direct bone formation measures, while no significant weight differences were noted. We conclude that chronic ethanol associated inhibition of direct bone formation is mediated to a significant extent by the TNF signaling axis in a mouse model.

Keywords

mouse; distraction osteogenesis; TNF; bone formation; ethanol

Introduction

Distraction Osteogenesis (DO) is a clinical method of direct bone formation and has been used both experimentally and clinically. DO is induced by gradually pulling apart the edges of a bone fracture (distraction), using an external fixator, to permit formation of new bone in the slowly expanding gap. New bone formation (direct, intramembranous, appositional) during

Correspondence should be addressed to: Dr. C. K. Lumpkin Jr, ACHRI, Slot 512-20B, 1120 Marshall Street, Little Rock, AR, 72202. Phone (501) 364-2797, Fax (501) 364-5880, e-mail: lumpkincharlesk@uams.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

DO is well organized and during the early phases is spatially isolated from the process of bone resorption.

Tumor necrosis factor- α (TNF) is a pro-inflammatory cytokine that plays an essential role in modulating both osteoclasts and osteoblasts. Previous studies have demonstrated the ability of TNF to block multiple osteoblast functions *in vitro*, as well as bone formation/repair *in vivo* (Nanes, 2003). Though high levels of TNF are known to inhibit osteoblastogenesis in culture and *in vivo*; nevertheless, low doses can enhance osteoblast proliferation in culture and impaired osteoblastogenesis has been demonstrated in TNFR1/R2 double knockout mice (Frost et al., 1997; Gerstenfeld et al., 2001). This suggests that normal expression of TNF is required for optimal bone formation but that unregulated or excessive expression results in pathology.

Previous studies have shown that alcohol abuse is correlated with osteoporosis, decreased bone mass, risk of fractures, and impaired fracture healing (Holden, 1987; Purohit, 1997). One characteristic of osteoporosis is a relative impairment in osteoblastogenesis. The DO model provides the opportunity to isolate and study ethanol's effects on osteoblastogenesis. DO studies using total enteral nutrition in the rat have demonstrated that chronic ethanol exposure decreases tibial bending strength, inhibits bone formation (osteoblastogenesis) during DO, and increases the expression of interleukin 1 (IL-1 β) and TNF in the liver, all in the context of optimal nutrition (Brown et al., 2002a,b; Perrien et al., 2002; Perrien et al., 2003; Wahl et al., 2005). Further, treatment of chronic ethanol exposed rats with a TNF receptor antagonist restores bone formation, while treatment of non-ethanol exposed rats with recombinant rat TNF (rrTNF) inhibits bone formation during DO (Brown et al., 2002b; Perrien et al., 2004; Wahl et al., 2005). In addition, several recent studies have used liquid diets to study the negative effects of chronic ethanol exposure on skeletal parameters in both rats and mice (Dai et al., 2000; Zhang et al., 2002; Chakkalakal et al., 2005; Chakkalakal, 2005). Recently, the combination of ethanol delivery by liquid diet with a unique mouse DO model has demonstrated the expected osteoinhibition of bone formation during DO (Aronson et al., 2002; Wahl et al., 2006). The establishment of this model in the mouse allows for expanded testing of mechanistic hypotheses and potential therapeutics associated with the inhibition of direct bone formation by chronic ethanol exposure.

The above results have led to the investigations reported here employing the mouse DO/liquid diet model. This report presents the results from experiments on 1) the effects of systemic delivery of a TNF receptor antagonist during DO to mice chronically exposed to ethanol, and 2) the effects of systemic administration of recombinant mouse TNF (rmTNF) on new bone formation during DO. We hypothesized that sTNFR1 would block the osteoinhibitive effects of ethanol and that rmTNF would inhibit direct bone formation during DO in ethanol naïve mice.

Materials and Methods

Animals

Virus-free adult male C57BL/6 mice were purchased from Harlan Industries (Indianapolis, IN). 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 5-7 days prior to surgery. In both studies, the mice were assigned to respective experimental groups with mean body weights equal to that of the control group (± 4 g) for the study, and the mice were weighed twice a week thereafter. All research protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arkansas for Medical Sciences.

Study Designs

Study 1: sTNFR1 + EtOH exposure + DO In the first study, forty-eight (48) C57BL/6 male 2-month-old mice were acclimated to the Lieber-DeCarli liquid control diet #710027 (Dyets) over a four day period. The mice were acclimated to the diet by adding a feeding tube of the liquid diet to the cage along with the pellet chow for three days, and then removing the pellet chow. The mice were separated into two diet groups: control (n=16) and ethanol (EtOH, n=32). The EtOH diet used was the Lieber-DeCarli liquid EtOH diet #710260 from Dyets. The two diets are designed to be isocaloric at 1.0 kcal/ml with a maximum ethanol concentration of 36% total calories. Our data demonstrate an average of 12.3 mls consumed per day, so the mean caloric intake is 12.3 kcal/day.

The control mice were group-fed to the EtOH mice by using the average mls of diet consumed by all of the EtOH mice. This group fed protocol, rather than the previous pair-fed protocol, was used to avoid weight loss of control mice due to distress and subsequent sacrifice of their paired EtOH partner. Every day we measured the average amount of diet the EtOH mice consumed and then gave that amount (but using control diet) to all of the control mice. The EtOH group started on 1% EtOH on the fifth day of the study. The EtOH was increased to 2% on day 7, to 3% on day 9, to 4% on day 11, and to 5% on day 13. The EtOH content stayed at 5% for the remainder of the study. After being on diet for 51 days, all mice, under Nembutal anesthesia, underwent placement of an external fixator and osteotomy on the left tibia (Aronson et al., 2002; Wahl et al., 2006). Buprenex (1.0 mg/kg) was given by intramuscular injection post surgery for analgesia. The surgeries were completed over an eight-day period. At this time the EtOH group had been receiving EtOH for 47 days. At the time of surgery, the EtOH mice were grouped into either an EtOH+vehicle (n=16) or an EtOH+sTNF-R1 (n=16) group. Post surgery, the mice received a subcutaneous injection of either vehicle (PBS pH 7.6), or sTNF-R1 (polyethylene glycol-r-met-sTNFR1, Amgen, 8.0 mg/kg) in PBS pH 7.6. These injections were then administered every other day for the remainder of the study. The mice in the control diet group were injected with the vehicle every other day. The dose and scheduling came from dose responses in rat studies both 1) in our lab with ethanol and DO studies and 2) in other labs with Type 1 osteoporosis and arthritis studies (Brown et al., 2002b; Perrien et al., 2004; Wahl et al., 2005). Following a three-day latency period, distraction began at a rate of 0.075mm twice a day (b.i.d.) for 14 days. At sacrifice trunk blood was collected, the left tibias and contra-laterals were placed in formalin, and the femur marrow was removed for flow cytometry and RT-PCR. Serum was obtained for ethanol measurement. The study ended with n=15 in the control group, n=15 in the EtOH+veh group, and n=14 in the EtOH+sTNF-R1 group.

Study 2 = rmTNFalpha + DO—In the second study, n=19 C57BL/6 12-week-old male mice (average weight 25.5g) were divided into two groups: control group (n=10) and rmTNF- α treated group (n=9). All mice were handled by animal care personnel seven days prior to the surgeries. Following acclimation and under nembutal anesthesia, each mouse underwent placement of a titanium ring fixator on the left tibia. Fourteen day, 100ul Alzet pumps (Model 1002) were placed subcutaneously on the back for systemic delivery of rmTNF- α (10 μ g/kg/day) or vehicle (phosphate buffered saline, pH 7.4). Buprenex (1.0 mg/kg) was given by intramuscular injection post surgery for analgesia. Distraction began 3 days after surgery (3 day latency) at a rate of 0.075 mm b.i.d. (0.15 mm/day) and continued for 14 days. All mice were sacrificed on postoperative day 17, at which time the distracted and contralateral tibiae and trunk blood were harvested. The blood was assayed for serum TNF- α measurement by bead array as below.

Distraction Protocol

Following acclimation and under Nembutal anesthesia, each mouse underwent placement of an external fixator and osteotomy to the left tibia. 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 (1.0mg/kg) was given by intramuscular injection post surgery for analgesia. Distraction began three days after surgery (3 day latency) at a rate of 0.075 mm b.i.d. (0.15 mm/day) and continued for 14 days. After the distraction period, the mice were sacrificed and the distracted tibiae were harvested for radiographic and histological analyses. The mice were weighed twice a week during the study.

Radiographic and Histological 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. 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 (1.25 × objective) microscopic magnification and the area and density 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 outlining the regions with radio density equivalent to or greater than the adjacent medullary bone. 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 (Aronson et al., 1997a,b; Skinner and Fromowitz, 1989).

After radiography, the distracted tibiae were decalcified in 5% formic acid, dehydrated, and embedded in paraffin. Experience in our laboratory has demonstrated that this achieves good morphology in murine orthopedic tissues and does not appear to significantly impair immunological detection of many epitopes (Aronson et al., 1997; Perrien et al., 2002). 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. As detailed above, a quadrilateral region of interest was outlined and recorded. 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 (Aronson et al., 1997a; Perrien et al., 2002, 2003; Skinner and Nicholas, 1990; Skinner et al., 1993, 1997; Wahl et al., 2006;). We note that the “fibrous interzone” that lies between the proximal and distal new bone formation fronts (i.e. the non-new bone gap area) is comprised of fibroblastic cells overlaying parallel collagen bundles which are oriented along the distraction vector.

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 no bone chips within the DO gap, 4) have an intact ankle, and 5) have had no weight loss or health problems during the distraction period.

RNA extraction and Semi-quantitative RT-PCR

Total RNA was prepared from femoral bone marrow using Tri Reagent (Molecular Research Center, Inc.). Briefly, RNA was extracted by adding 1/5 (v/v) chloroform into the mixture of Tri-reagent and cells. RNA was precipitated after mixing with an equal amount of isopropanol and washed twice in 75% ethanol. Finally, total RNA was dissolved in DEPC-H₂O and stored in -75°C. The RNA was analyzed in 1% agarose gel and treated with RQ1 RNase-free DNase (Promega). Then, the RT-PCR was performed following the instructions provided by the manufacturer (Life Technologies). Briefly, first-strand cDNA was synthesized by using THERMOSCRIPT™ Reverse Transcription System (Life Technologies) with DNase-treated total RNA. Reverse transcriptase was left out of one sample as a negative control. PCRs were conducted using the GeneAmp PCR System 9600 (Perkin Elmer, USA). The PCR products were analyzed using a Gel Doc 1000 (BIO-RAD) and Molecular Analyst™ software (BIO-RAD). The specific primers used for PCR are summarized in the Table 1. The primers specific for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene were used to equalize the amount of cDNA in the each sample. Every RT-PCR was repeated at least twice with the similar results.

Serum mouse TNF α Bead Array

Serum samples were run on the Becton Dickinson FACS Calibur per the manufacturer's instructions in the UAMS Core facility. The mouse TNF1/TH2 kit was used.

Serum ethanol concentrations

The serum ethanol concentration of each sample was determined using a GL5 Analox analyzer (Analox Instruments Limited, London, UK) according to the manufacturers directions as previously described (Brown et al., 2002a).

Statistics

For statistical analysis, differences between group means were determined by the Student's t test or by One-Way ANOVA. Also, the Student-Newman-Keuls Method was used for the pairwise multiple comparisons and the p values agreed with the ANOVA values. All data are reported as mean $\pm$ standard error of the mean (SEM). Differences were considered significant when $P < 0.05$.

Results

Study 1

To study the effects of a TNF receptor antagonist given during DO after chronic ethanol exposure forty-eight C57BL/6 male 2-month-old mice were acclimated to the Lieber-DeCarli liquid control diet and then the mice were separated into two diet groups: control (n=16) and EtOH (n=32). After being on liquid diet for 51 days, all mice underwent placement of an external fixator and osteotomy on the left tibia. At the time of surgery, the EtOH mice were grouped into either an EtOH+vehicle (n=16) or an EtOH+sTNF-R1 (n=16) group. All mice then underwent the DO protocol.

The average serum ethanol concentration in the EtOH group at sacrifice (8:00-10:00 AM) was 73 ± 17 mg/dl (range = 21-152), which is in agreement with previous reported termination values (Chakkalakal et al., 2005; Dai et al., 2000; Wahl et al., 2006). All mice were weighed

throughout the study and no significant differences in body weight gains or final weights (EtOH: 27.3 ± 1.9 vs group-fed: 28.3 ± 1.7 g) were noted (Figure 1). In fact, the average weight gain per week before surgery in both groups was 0.59 grams/week which is not significantly different from 0.6 grams/week, which is the value resulting from ad lib chow feeding of same age mice taken from the C57BL/6 mouse growth curves supplied by Harlan, as well as published data from this lab (Wahl et al., 2006). These results support the lack of confounding nutritional effects in the liquid diet mouse model, in either the EtOH or the group-fed mice, and are consistent with recent reports (Dai et al., 2000; Zhang et al., 2002; Chakkalakal et al., 2005; Chakkalakal et al., 2005; Wahl et al., 2006).

New gap bone formation after DO in mice group-fed control or EtOH diets was assessed by single beam radiography and histology. Comparison of the distracted tibial radiographs demonstrated 1) the expected ethanol-associated inhibition of percent new bone formation/mineralization (group-fed $59.5\% \pm 6.6$ vs EtOH $26.7\% \pm 6.7$, $p=.002$), and 2) the positive effects of sTNFR1 treatment [sTNFR1 treated EtOH $52.3\% \pm 5.6$ vs vehicle treated EtOH $26.7\% \pm 6.7$, $p=.009$] (Figures 2, 4).

Further, analysis of histological sections supported the radiological analyses by revealing a significant decrease in new gap bone formation in EtOH ($21.2 \pm 5.5\%$) vs both group-fed ($58.6 \pm 7.1\%$, $p=.002$) and sTNFR1 treated EtOH mice ($54 \pm 5.9\%$, $p=.002$) (Figures 3,4).

Representative H&E stained histological sections of distracted tibial DO gaps from group-fed, EtOH-fed, and sTNFR1 treated EtOH mice are shown in Figure 3. The area of new gap bone formation is roughly outlined with dashed lines for clarification. Notice the significant reduction in the area of new bone formation in the EtOH specimen in comparison to the pair-fed and sTNFR1 treated specimens.

RT-PCR performed on bone marrow derived mRNA revealed significant EtOH associated increases in CYP 2E1 ($p<.001$), TNF α ($p<.01$), IL-6 ($p<.05$), but not IL-1 β (Figures 5, 6; Table 1).

Study 2

To study the effects of TNF on DO in non-ethanol exposed mice, nineteen C57BL/6 male mice underwent the standard DO protocol with the addition of the placement of alzet pumps subcutaneously mid back. The pumps delivered 10 μ g/kg/day rmTNF ($n=15$) or vehicle ($n=12$) over the first 14 days of the 17 day distraction protocol (3 day latency, 14 day lengthening @ .075 mm bid). Weight changes were equivalent in both groups. The pre-surgery weights were 25.2 ± 0.2 g for TNF treated vs 25.9 ± 0.3 g for vehicle controls; while the harvest weights were 25.4 ± 0.3 g for TNF treated vs 25.6 ± 0.3 for controls. Increased circulating levels of serum TNF- α in treated mice confirmed the delivery of rmTNF by alzet pump (13.05 ± 5.99 pg/ml in treated vs undetected in controls, $p<0.01$).

Radiographically, the percent of mineralized new bone per gap was $59.5\% \pm 3.9$ in the vehicle group vs $38.8\% \pm 3.5$ in the rmTNF- α group, $p<0.001$ (Figures 7,9). Comparison of the distracted tibial histology also demonstrated significant decreases in new gap bone formation in the rmTNF treated group ($29.3 \pm 5.5\%$: rmTNF vs $69.6 \pm 4.4\%$ vehicle, $P<.001$, Figures 8,9). Representative histological sections of distracted tibiae from rmTNF treated mice and vehicle treated mice are shown in Figure 8. The area of new bone formation is roughly outlined with dashed lines for clarification. Notice the significant reduction in the area of new bone formation in the rmTNF treated specimen in comparison to the vehicle treated control.

Discussion

Previous studies using total enteral nutrition in the rat have demonstrated that chronic ethanol exposure decreases tibial bending strength, and inhibits bone formation (osteoblastogenesis) during DO; while treatment with a TNF receptor antagonist normalizes bone formation during DO, all in the context of optimal nutrition (Brown et al., 2002a,b; Perrien et al., 2002; Perrien et al., 2003; Wahl et al., 2005). Further, treatment of non-ethanol exposed rats with recombinant rat TNF (rrTNF) inhibits bone formation during DO (Brown et al., 2002b; Wahl et al., 2005). Taken together, these studies suggest a role for the TNF signaling axis in the inhibitory effects of chronic ethanol exposure on direct bone formation, at least in this specific rat model. Development of a mouse model for chronic ethanol studies on DO could be helpful to further mechanistic studies in this area. In this regard, studies have used liquid diets to successfully study the negative effects of chronic ethanol exposure on skeletal parameters in both rats and mice (Dai et al., 2000; Zhang et al., 2002; Chakkalakal et al., 2005; Chakkalakal, 2005). Most recently, the combination of ethanol delivery by liquid diet with the mouse DO model, utilized in this report, has demonstrated the osteoinhibition of bone formation during DO equivalent to that seen in the enteral rat model (Aronson et al., 2002; Wahl et al., 2006).

Collectively, the above results have led to the investigations reported here employing the mouse DO/liquid diet model for the chronic ethanol experiment. This report presents the results from experiments on 1) the effects of systemic delivery of a TNF receptor antagonist during DO to mice chronically exposed to ethanol, and 2) the effects of systemic administration of recombinant mouse TNF α (rmTNF) on new bone formation in ethanol naive mice during DO. We demonstrate in this report that sTNFR1 blocks the osteoinhibitive effects of ethanol exposure in mice and that rmTNF inhibits direct bone formation during DO in ethanol naive mice.

In Study 1, where the effects of a TNF receptor antagonist on DO in chronic ethanol exposed mice were determined, the findings of no significant differences in the weight gains or weight recovery reported here support the lack of confounding nutritional effects in the liquid diet mouse DO model, in either the EtOH or the group-fed mice, and are consistent with recent reports (Dai et al., 2000; Zhang et al., 2002; Chakkalakal et al., 2005; Chakkalakal, 2005; Wahl et al., 2006). The radiologic value of 59.5% and the histologic value of 58.6% new gap bone formation for the group-fed mice are not significantly different from values from two other unpublished studies of the same mouse strain fed chow ad lib (51%/52%, and 58%/65%). Though caution must be exercised in cross study comparisons, these values also support the lack of nutritional deficits in the group-fed mice as opposed to chow ad lib fed mice in regard to new gap bone formation. Taken together, these results demonstrate that the amount of ethanol needed to produce skeletal toxicity is below levels that produce reduced food intake and that liquid diets can be an effective means of ethanol/diet delivery to study ethanol related skeletal pathology.

The inhibition by chronic ethanol exposure of new gap bone formation during DO in mice reported here is of a similar magnitude as that caused by chronic ethanol exposure and aging during DO in both rats and mice (Aronson et al., 2001, 2002; Brown et al., 2002a,b; Wahl et al., 2006). The effects on new bone formation during DO demonstrated here are in agreement with a recent paper, which used the same liquid diet (36 % total calories as ethanol) and demonstrated that bone repair in rats was also compromised due to the ethanol alone and not to the nutritional variables (Chakkalakal, 2005; Chakkalakal et al., 2005). The rat bone repair model in this paper was a fibular 4 mm gap osteotomy which heals similarly to other fracture models by both direct and endochondral bone formation. It is probable that the direct bone formation in this rat model is similar if not identical to that studied here in mice during distraction.

The normalization of direct bone formation associated with the administration of the TNF receptor antagonist is also in agreement with previous reports which employed rats, enteral ethanol/diet, and DO or fracture (Brown et al., 2002b; Perrien et al., 2004; Wahl et al., 2005). The similarity between these findings in rat and mouse suggests 1) that both species respond to chronic ethanol exposure with similar mechanisms, and 2) that delivery of ethanol in mice by liquid diet approximates the nutritional support characteristic of the complete enteral nutritional delivery used with the rats.

The findings of ethanol induced mRNA levels in the bone marrow of the CYP 2E1, TNF α , and IL-6 genes are consistent with many other ethanol studies on the effects of ethanol on the liver. These findings are also consistent with the hypothesis that since TNF α appears to be involved in the inhibitory effects of chronic ethanol exposure in this model system. Based on results from hepatotoxicity studies another hypothesis is that the ethanol induction of CYP2E1 in the marrow leads directly to increased levels of reactive oxygen species (**ROS**) which precede the induction of TNF α in several organ systems (Ronis et al., 1993; Badger et al., 1993). We have no direct evidence of ROS alone inducing TNF; however, we do have evidence that TNF induction by ethanol in bone marrow is abolished by treatment with dietary antioxidant (NAC) (Shankar et al, 2006). This is consistent with, but not proof of, ROS-induced TNF. We note that the induction in the marrow of CYP2E1 by ethanol may be in non-osteoblastic cells as a recent report failed to find CYP2E1 induction in cultured osteoblasts but did demonstrate induction of class I alcohol dehydrogenase (Chen et al., 2006). Another possibility that has been suggested by some investigators, mostly using rat models, is that ethanol in a liquid diet decreases intestinal barrier function leading to increased uptake of LPS or other bacterial components that can induce TNF (Thakur V et al., 2006). We believe that these mechanisms underlying induction of TNF associated with chronic ethanol exposure are not mutually exclusive.

In Study 2, the inhibition by rmTNF of new gap bone formation during DO in ethanol naïve mice reported here is of a similar magnitude to that caused by administration of rrTNF to ethanol naïve rats during DO (Wahl et al., 2005). That these effects are seen without significant changes in body weights between the treated and control groups suggest that the dose of rmTNF was appropriate to study skeletal toxicity without confounding nutritional variables and is in agreement with a previous dose study (Hashimoto et al., 1989). As opposed to the rat study, the mouse DO gaps treated with TNF did not contain areas of inflammation likely due to the difference in delivery, i.e. local extraperiosteal (rat) vs systemic (mouse). We believe the systemic route may be more appropriate as an analog to human ethanol exposure; however, local infusion may facilitate future/alternate studies (Fowlkes et al., 2006). These results suggest that TNF has similar osteoinhibitive properties during DO in the mouse as it does in the rat, and this supports the use of this model to compare the effects of ethanol to systemic rmTNF delivery in regard to a number of postulated mechanistic variables.

In conclusion, the data suggest that osteogenesis in both rats and mice respond to chronic ethanol exposure in a similar manner and that liquid ethanol diet delivery can be used in mice to study the underlying mechanisms. These and other studies set the stage for using the ever widening array of genetically altered mouse strains to study the negative effects of chronic ethanol exposure on direct bone formation during DO and fracture healing. Further, the mouse DO model can be used to study the effects of excess TNF α administration on direct bone formation, a situation common to other pathologies such as rheumatoid arthritis and aging. For future studies, we postulate that chronic high ethanol consumption results in local elevations of TNF activities, which inhibit osteoblastogenesis at multiple stages during DO. The goals of this research are to support pharmacological and/or nutritional interventions in orthopaedic procedures with the focus on non-unions and delayed unions in patients with alcohol and cytokine associated bone loss.

Acknowledgements

Supported by NIH grant AA12223 (CKL), by NIH National Center for Research Resources Grant # 1CORR16517-01, and by Arkansas Biosciences Institute (CKL) funded by the Arkansas Tobacco Settlement Plan and administered by Arkansas Children's Hospital Research Institute.

References

1. Aronson J, Shen X, Gao GG, Miller F, Quattlebaum T, Skinner RA, Badger TM, Lumpkin CK Jr. Sustained proliferation accompanies distraction osteogenesis in the rat. *J. Orthop. Res* 1997;15(a): 563–569. [PubMed: 9379266]
2. Aronson J, Shen X, Skinner RA, Badger TM, Lumpkin CK Jr. Rat model of distraction osteogenesis. *J. Orthop. Res* 1997;15(b):221–226. [PubMed: 9167624]
3. Aronson J, Gao GG, Shen X, McLaren SG, Skinner RA, Badger TM, Lumpkin CK Jr. Effect of aging on distraction osteogenesis in the rat. *J. Ortho. Res* 2001;19:421–427.
4. Aronson J, Liu L, Liu Z, Gao GG, Perrien D, Brown E, Skinner RA, Thomas JR, Morris KD, Suva LJ, Badger TM, Lumpkin CK Jr. Decreased endosteal membranous bone formation accompanies aging in a mouse model of distraction osteogenesis. *e-biomed. J. Regenerative Med* 2002;3:7–16.
5. Badger TM, Huang J, Ronis MJJ, Lumpkin CK Jr. Induction of cytochrome p450 2E1 during chronic ethanol exposure occurs via transcription of the CYP 2E1 gene when blood alcohol concentrations are high. *Biochem Biophys Res Com* 1993;190:780. [PubMed: 8439329]
6. Brown EC, Perrien DS, Fletcher TW, Irby DJ, Aronson J, Gao GG, Hogue WR, Skinner RA, Suva LJ, Ronis MJJ, Hakkak R, Badger TM, Lumpkin CK Jr. Skeletal toxicity associated with chronic ethanol exposure in a rat model utilizing total enteral nutrition. *J. Pharmacol. Exp. Ther* 2002;301(a):1132–1138. [PubMed: 12023547]
7. Brown EC, Perrien DS, Fletcher TW, Irby DJ, Aronson J, Gao GG, Hogue WR, Skinner RA, Feige U, Suva LJ, Ronis MJJ, Badger TM, Lumpkin CK Jr. Il-1 and TNF antagonists attenuate ethanol-induced inhibition of bone formation in a rat model of distraction osteogenesis. *J. Pharmacol. Exp. Ther* 2002;303(b):904–908. [PubMed: 12438508]
8. Chakkalakal DA. Alcohol-induced bone loss and deficient bone repair. *Alcohol Clin. Exp. Res* 2005;29:2077–2090. [PubMed: 16385177]
9. Chakkalakal DA, Novak JR, Fritz ED, Mollner TJ, McVicker DL, Garvin KL, McGuire MH, Donohue TM. Inhibition of bone repair in a rat model for chronic and excessive alcohol consumption. *Alcohol* 2005;36:201–214. [PubMed: 16377462]
10. Chen JR, Haley RL, Hidestrand M, Shankar K, Liu X, Lumpkin CK Jr, Badger TM, Ronis MJJ. Estradiol protects against ethanol-induced bone loss by inhibiting up regulation of RANKL in osteoblasts. *J Pharmacol Exp Ther.* 2006in press
11. Dai J, Lin D, Zhang J, Habib P, Smith P, Murtha J, Fu Z, Yao Z, Qi Y, Keller E. Chronic alcohol ingestion induces osteoclastogenesis and bone loss through Il-6 in mice. *J. Clin. Invest* 2000;106:887–895. [PubMed: 11018077]
12. Fowlkes JL, Thraikill KM, Liu L, Wahl EC, Bunn RC, Cockrell G, Perrien DS, Aronson J, Lumpkin CK Jr. Effect of local administration of recombinant human IGF-1 on bone formation in an aged mouse model. *J Bone Miner Res* 2006;21:1359–1366. [PubMed: 16939394]
13. Frost A, Jonsson K, Nilsson O, Ljunggren O. Inflammatory cytokines regulate proliferation of cultured human osteoblasts. *Acta. Ortho. Scand* 1997;68(2):91–96.
14. Gerstenfeld L, Cho T, Kon T, Aizawa T, Cruceta J, Graves B, Einhorn T. Impaired intramembranous bone formation during bone repair in the absence of tumor necrosis factor-alpha signaling. *Cells Tissues Organs* 2001;169:285–294. [PubMed: 11455125]
15. Hashimoto J, Yoshikawa H, Takaoka K, Shimizu N, Masuhara K, Tsuda T, Miyamoto S, Ono K. Inhibitory effects of tumor necrosis factor alpha on fracture healing in rats. *Bone* 1989;10:453–457. [PubMed: 2624827]
16. Holden C. Alcoholism and the medical cost crunch. *Science* 1987;235:1132–33. [PubMed: 3823872]
17. Nanes MS. Tumor necrosis factor- α : molecular and cellular mechanisms in skeletal pathology. *Gene* 2003;321:1–15. [PubMed: 14636987]

18. Perrien D, Brown EC, Aronson J, Skinner RA, Montague DC, Badger TM, Lumpkin CK Jr. Immunohistochemical study of osteopontin expression during distraction osteogenesis in the rat. *J. Histochem. Cytochem* 2002;50(4):567–574. [PubMed: 11897810]
19. Perrien DS, Liu Z, Wahl EC, Bunn RC, Skinner RA, Aronson J, Fowlkes J, Badger TM, Lumpkin CK Jr. Chronic ethanol exposure is associated with a local increase in TNF α and decreased proliferation in the rat distraction gap. *Cytokine* 2003;23:179–189. [PubMed: 12967643]
20. Perrien DS, Wahl EC, Hogue WR, Feige U, Aronson J, Ronis MJJ, Badger TM, Lumpkin CK Jr. IL-1 and TNF antagonists prevent inhibition of fracture healing by ethanol in rats. *Toxicol. Sci* 2004;82:656–660. [PubMed: 15470231]
21. Purohit V. Introduction to the alcohol and osteoporosis symposium. *Alcohol Clin. Exp. Res* 1997;21:383–384.
22. Ronis MJJ, Crouch J, Mercado C, Irby D, Valentine CR, Lumpkin CK Jr. Ingelman-Sundberg M, Badger TM. Cytochrome p450 CYP 2E1 induction during chronic alcohol exposure occurs by a two step mechanism associated with blood alcohol concentration in rats. *J Pharmacol Exp Ther* 1993;264:944. [PubMed: 8437134]
23. Shankar K, Hidestrand M, Liu X, Chen J-R, Perrien D, Skinner RA, Lumpkin CK Jr, Badger TM, Ronis MJJ. Ethanol-induced inhibition of anabolic rebuilding in post-weaning rats involves increased oxidative stress and TNF alpha in rats fed via total enteral nutrition. *Endocrine Soc. 2006abstract*
24. Skinner, RA.; Fromowitz, FB. Faxitron X-ray unit in histologic evaluation of breast biopsies. Tech. Sample HT-5. ASCP Press; Chicago, IL: 1989.
25. Skinner, RA.; Nicholas, R. Preparing superior quality slides of orthopedic tissue using previously decalcified material. Tech. Sample HT 2. ASCP Press; Chicago, IL: 1990.
26. Skinner RA, Nicholas RW, Stewart CL, Vireday C. Resected allograft bone containing an implanted expanded polytetrafluoroethylene prosthetic ligament: comparison of paraffin, glycolmethacrylate, and exakt grinding techniques. *J. Histotech* 1993;16:129–137.
27. Skinner RA, Hickmon SG, Lumpkin CK Jr. Aronson J, Nicholas RW. Decalcified bone: twenty years of successful specimen management. *J. Histotech* 1997;20:267–277.
28. Thakur V, Pritchard MT, McMullen MR, Wang Q, Nagy LE. Chronic ethanol feeding increases activation of NADPH oxidase by lipopolysaccharide in rat Kupffer cells: role of increased reactive oxygen in LPS-stimulated ERK1/2 activation and TNF-alpha production. *J Leukoc Biol* 2006;79:1348–56. [PubMed: 16554353]
29. Wahl EC, Perrien DS, Aronson J, Liu Z, Fletcher TW, Skinner RA, Feige U, Suva LJ, Badger TM, Lumpkin CK Jr. Ethanol-induced inhibition of bone formation in a rat model of distraction osteogenesis: A role for the tumor necrosis factor signaling axis. *Alcohol Clin. Exp. Res* 2005;29:1466–1472. [PubMed: 16131855]
30. Wahl EC, Liu L, Perrien DS, Aronson J, Hogue WR, Skinner RA, Hidestrand M, Ronis MJJ, Badger TM, Lumpkin CK Jr. 2006. A novel mouse model for the study of the inhibitory effects of chronic ethanol exposure on direct bone formation. *Alcohol*. 2006(in press)
31. Wahl EC, Aronson J, Skinner RA, Lumpkin CK Jr. Brdu Tracking Of Pre-Osteoblasts In Formalin Fixed, Decalcified Regenerating Bone. *J. Histotechnol* 2006;29:11–16.
32. Zhang J, Dai J, Lin D, Habib P, Smith P, Murtha J, Fu Z, Yao Z, Qi Y, Keller E. Osteoprotegerin abrogates chronic alcohol ingestion-induced bone loss in mice. *J. Bone Miner. Res* 2002;17:1256–1263. [PubMed: 12096839]

Figure 1.

All mice were weighed throughout the study and no significant differences in body weight gains or final weights (EtOH: 27.3 ± 1.9 vs group-fed control: 28.3 ± 1.7 g) were noted. Liquid diets were begun on week 2 and after 51 days on liquid diet all mice underwent surgeries on weeks 8 - 9. The average weight gain per week before surgery in both groups was 0.59 grams/week which is not significantly different from the value (0.6 grams/week) resulting from ad lib chow feeding of same age mice taken from the C57BL/6 mouse growth curves supplied by Harlan, as well as published data from this lab (Wahl EC et al, in press).

Figure 2. New gap bone formation after DO in mice group-fed control or EtOH diets $\pm$ sTNFR1 was assessed by single beam radiography. Comparison of the distracted tibial radiographs demonstrated ethanol-associated inhibition of % new bone formation/mineralization and the positive effects of sTNFR1 treatment. The pictures are aligned top to bottom/proximal to distal and the average external cortical diameter is ~ 1 mm.

Figure 3.

Representative H&E stained histological sections of distracted tibial DO gaps from group-fed control, EtOH-fed, and sTNFR1 treated EtOH mice are shown. The area of new gap bone formation is roughly outlined with dashed lines for clarification. Notice the significant reduction in the area of new bone formation in the EtOH specimen in comparison to the group-fed control and sTNFR1 treated specimens. **The pictures are aligned top to bottom/proximal to distal and the average external cortical diameter is ~1mm.**

Figure 4.

New gap bone formation after DO in mice group-fed control or EtOH diets $\pm$ sTNFR1 was assessed by single beam radiography and histology. Comparison of the distracted tibial radiographs demonstrated 1) ethanol-associated inhibition of % new bone formation/mineralization (**group-fed control > EtOH, $p=.002$**), and 2) the positive effects of sTNFR1 treatment [**sTNFR1 treated EtOH > vehicle treated EtOH, $p=.009$**]. Analysis of histological sections supported the radiological analyses by revealing a significant decrease in new gap bone formation in **EtOH < both group-fed control ($p=.002$) and sTNFR1 treated EtOH ($p=.002$)**.

Figure 5. Photograph of gel with RT PCR bands from mRNA derived from the bone marrow of ethanol and control fed mice. Gene expression was measured for the following genes: CYP 2E1, TNF α , IL-1 β , IL-6, and GAPDH (loading control).

Figure 6.

Quantitation of the RT PCR gel scan. RT-PCR performed on bone marrow derived mRNA revealed significant EtOH associated increases in CYP 2E1 ($p < .001$), TNF α ($p < .01$), IL-6 ($p < .05$), but not IL-1 β .

Figure 7. New gap bone formation after DO in mice $\pm$ rmTNF alpha was assessed by single beam radiography. Comparison of the distracted tibial radiographs demonstrated rmTNF-associated inhibition of % new bone mineralization. The pictures are aligned top to bottom/proximal to distal and the average external cortical diameter is ~ 1 mm.

Figure 8. Representative histological sections of distracted tibiae from rmTNF treated mice and vehicle treated mice are shown. The area of new endosteal bone formation is roughly outlined with dashed lines for clarification. Notice the significant reduction in the area of new bone formation in the rmTNF α treated specimen in comparison to the vehicle treated control. **The pictures are aligned top to bottom/proximal to distal and the average external cortical diameter is ~1mm.**

Figure 9.

New gap bone formation after DO in mice treated with rmTNF α or vehicle was assessed by single beam radiography and histology. Radiographically, the percent of mineralized new bone per gap was greater **in the vehicle group vs the rmTNF- α group ($p < 0.001$)**. Comparison of the distracted tibial histology also demonstrated decreases in new gap bone formation **in the rmTNF treated group ($p < .001$)**.

The specific primers used in RT-PCR analysis

Genes	Orientation	Sequence	Size	Annealing	Cycles
TNF- α Acc.No:U00981	Sense Antisense	5'-cgctcttctgtctactgaac-3' 5'-ttctccagctggagactcc-3'	540 bp	57°C	33
IL-1 β Acc.No:NM008361	Sense Antisense	5'-caggatgagagacatgagcacc-3' 5'-ctctgcagactcaactccac-3'	447 bp	60°C	31
IL-6 Acc.No:M26745	Sense Antisense	5'-cttccctacttccacaagtc-3' 5'-gaccacagtgaggaatgcc-3'	650 bp	57°C	32
Cyp2E1 Acc.No:NM031543	Sense Antisense	5'-ctgattggctgcgaccctgc-3' 5'-gaacaggctcgcccaagtcac-3'	381 bp	67°C	35
GAPDH Acc.No:AF106860	Sense Antisense	5'-ccctctggaagctgtggcgt-3' 5'-ttggaggcccatgtagccat-3'	431 bp	60°C	29