0022-3565/02/3033-904–908$7.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 303, No. 3
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39636/1024591
JPET 303:904–908, 2002
Printed in U.S.A.

Interleukin-1 and Tumor Necrosis Factor Antagonists Attenuate Ethanol-Induced Inhibition of Bone Formation in a Rat Model of Distraction Osteogenesis

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Received June 25, 2002; accepted August 15, 2002

ABSTRACT

Chronic ethanol exposure inhibits rapid bone formation during distraction osteogenesis (DO; fracture and limb lengthening) and decreases volumetric bone mineral density (BMD) in a model of intragastric dietary infusion [total enteral nutrition (TEN)] in the rat. The hypothesis tested herein was that overexpression of interleukin (IL)-1β and tumor necrosis factor (TNF)-α mediates these deleterious effects of ethanol on the rat skeleton. Two studies (study 1, female rats; study 2, male rats) were performed to test the potential protective effects of the IL-1 and TNF antagonists: IL-1 receptor antagonist (IL-1ra) and 30-kDa polyethylene glycol-conjugated soluble TNF receptor type 1 (sTNFR1). All rats were infused with a liquid diet ± ethanol (EtOH) and underwent tibial fractures and DO. During distraction, the animals received a combination of IL-1ra (1.8–2.0 mg/kg/day) and sTNFR1 (2.0 mg/kg/2 days) or vehicle. A comparison of distracted tibial histological sections demonstrated 1) significant antagonist-related increases in bone column formation over the EtOH controls (studies 1 and 2), and 2) restoration of new bone equivalent to that of the TEN controls (study 2). In contrast, examination of intact proximal tibial metaphyses by peripheral quantitative computerized tomography revealed decreases in volumetric BMD of both EtOH control and EtOH antagonist groups (study 2). These results demonstrate that short-term systemic administration of IL-1 and TNF antagonists together protect rapid bone formation during DO from the deleterious effects of chronic ethanol but are ineffective in regard to intact bone homeostasis.

Excessive alcohol consumption has been reported to interfere with human bone homeostasis and repair in multiple ways, and these studies are summarized in a recent review (Purohit, 1997). Relevant herein, patients with alcohol-induced bone disease display marked impairment in bone formation (Crilly et al., 1988). Previous studies by these authors have shown that chronic ethanol exposure via an intragastric dietary delivery system [total enteral nutrition (TEN)] inhibits bone formation during distraction osteogenesis (DO) in the rat (Brown et al., 2002). This model replicates several important aspects of human alcoholic liver disease (Badger et al., 1993). DO is a unique clinical method of bone formation developed by Ilizarov and has been used both experimentally and clinically (Aronson, 1994). DO is induced by slowly pulling apart the edges of an intentionally introduced bone fracture, using an external fixator, to permit rapid formation of new bone in the slowly expanding gap (Aronson, 1994). New bone formation during DO is well organized and spatially isolated from the process of bone resorption. Several studies have demonstrated that the histological pattern of bone formation by DO in dogs, rabbits, rats, and mice is analogous to that in humans (Aronson, 1994; Tay et al., 1998; Aronson et al., 2001). In a manner comparable with humans, rats develop bone loss with increasing age, gonadectomy, and alcohol exposure (Jee, 1991; Sampson, 1998). This suggests that the rodent model of DO may hold clinical relevance for the investigation of ethanol-associated alterations in bone formation during DO.

This study was supported in part by National Institutes of Health Grants AA12223 and AA08645. Some of these data were reported in an abstract at the Society of Toxicology 2001 annual meeting (March 25–29; San Francisco, CA).

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Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
DOI: 10.1124/jpet.102.039636.

ABBREVIATIONS: TEN, total enteral nutrition; DO, distraction osteogenesis; BMD, volumetric bone mineral density; IL, interleukin; TNF, tumor necrosis factor; EtOH, ethanol; UEC, urinary ethanol content; sTNFR1, soluble tumor necrosis factor receptor type 1; IL-1ra, recombinant human interleukin-1 receptor antagonist; PBS, phosphate-buffered saline; veh, vehicle; pQCT, peripheral quantitative computed tomography.

Previous studies demonstrate that ethanol exposure decreases bone mineral density (BMD), inhibits bone formation during DO, and increases the expression of IL-1β and TNF-α in the liver and bone marrow (Fang et al., 1998; Turner et al., 1998; Brown et al., 2002). IL-1β and TNF-α are known to be potent inhibitors of bone formation in vitro and in vivo (Bertolini et al., 1986; Nguyen et al., 1991). Furthermore, inhibition of IL-1 and TNF activities has positive effects on bone formation in the ovariectomized rat that are independent of the inhibition of resorption (Kimble et al., 1995). Thus, the hypothesis for the studies reported herein is that systemic administration of IL-1 and TNF antagonists will attenuate the inhibitory effects of chronic ethanol on bone formation during DO and protect the BMD of intact bone in ethanol-exposed rats.

Materials and Methods

Animals. Virus-free adult Sprague-Dawley rats (3-month-old, 300-g females and 3-month-old, 350-g males) were purchased from Harlan (Indianapolis, IN). They were housed in individual cages in temperature-controlled (22°C) and humidity-controlled (50%) rooms having a 12-h light/dark cycle. All rats were handled by animal care personnel for 5 to 7 days before surgery. In both studies, the rats were assigned to respective experimental groups with mean body weights equal to that of the control group (± 4 g) for the study, and the rats were weighed twice a week thereafter. These studies were approved by the Institutional Animal Care and Use Committee. All procedures for DO (Aronson et al., 1997) and the TEN system (Badger et al., 1993; Brown et al., 2002) as well as the diet formulations (Lumpkin et al., 1996; Brown et al., 2002) have been described previously in detail.

Study 1. To study the potential roles of IL-1 and TNF in ethanol-related inhibition of bone formation during DO in female rats, 10 female Sprague-Dawley rats were randomly assigned to one of two groups, EtOH + vehicle or EtOH + antagonists. Intragastric cannulae were implanted in all animals for infusion of the liquid diet. Briefly, a small silicone cannula was inserted through the wall of the stomach and tunneled subcutaneously to the head. There, it was attached to a headpiece secured to the skull by four jeweler's screws and tethered to the top of the cage for infusion of the liquid diet. During the same surgical procedure, stainless steel ring fixators were applied to the left tibia in standardized manner (Aronson, 1994; Brown et al., 2002). All rats received 0.1 mg/kg Buprenex for analgesia and were returned to their cages for observation during recovery.

Dietary infusion began no sooner than 4 h after recovery from anesthesia. Sterile techniques were used to prepare all solutions, and diet was infused at a rate of 187 kcal/kg$^{0.75}$/day. Water was available ad libitum. After 1 week of acclimation to the TEN system (infusion of liquid diet without ethanol), all rats received increasing doses of EtOH (10–12 g/kg) in the liquid diet for 6 weeks. As ethanol was added, an isocaloric amount of carbohydrates was removed from the diet so that the caloric density of the diet was unchanged throughout the study. Urine ethanol concentrations (UECs), which correlate positively with blood alcohol content, were measured daily for the duration of the study, as described previously (Badger et al., 1993). The animals were maintained on the liquid diet containing ethanol (12 mg/kg/d) for 6 weeks before fracture of the fixated tibia and treatment with either IL-1ra and sTNFR1 or vehicle.

Briefly, under isoflurane anesthesia, all rats received left tibial fractures, as described previously (Aronson et al., 1997; Brown et al., 2002). Alzet mini-osmotic pumps (model 2002; Durect, Cupertino, CA) containing either IL-1ra (2.0 mg/kg/day) (Amgen, Thousand Oaks, CA) or vehicle were placed subcutaneously on the back. In addition, each rat received a subcutaneous injection of either 2.0 mg/kg sTNFR1 (Amgen) in PBS or PBS alone. After creation of the tibial fracture and placement of the osmotic pump, the rats were returned to their cages, and dietary infusion was resumed no sooner than 2 h after recover from anesthesia. The rats continued to receive 2.0 mg/kg sTNFR1 or PBS by subcutaneous injection every other day until sacrifice. Distraction began the day after fracture (1-day latency) at 0.2 mm b.i.d. (0.4 mm/day) and continued for 14 days. Immediately after the 14-day distraction period, rats were euthanized under anesthesia. The distracted tibiae were surgically removed, and the soft tissues dissected away.

Study 2. The following study was performed to determine the potential roles of IL-1 and TNF in ethanol-induced inhibition of bone formation during DO in male rats, and to compare the bone formation in the EtOH + antagonist and EtOH + veh groups to that in rats given a nonalcoholic diet. In addition, the effects of the IL-1 and TNF antagonists on ethanol-induced bone loss in the intact skeleton were examined in the nonfractured contralateral tibiae of these animals. Fifty male Sprague-Dawley rats were randomly assigned to one of three groups: control + veh, EtOH + veh, or EtOH + antagonist. All procedures for this study were identical to those in study 1 unless stated otherwise. All diets are liquid TEN diets ± ethanol. Thus, the composition of the control diet was exactly the same as the ethanol diet except that carbohydrates were isocalorically substituted for ethanol. After 4 days of recovery from placement of the intragastric cannula and external fixator and acclimation to the liquid diet, the EtOH + veh and EtOH + antagonist groups received increasing doses of EtOH (8–10.5 g/kg/day) in the liquid diet over an 11-day period and were maintained at 10.5 g/kg/day EtOH for the remainder of the study. UECs were measured daily for the duration of the study (Badger et al., 1993). After acclimation to ethanol and under isoflurane anesthesia, all animals underwent left tibial fractures and placement of Alzet mini-osmotic pumps (model 2004) containing either 100 mg/ml IL-1ra sufficient to deliver 1.8 mg/kg/day for EtOH + antagonist rats or vehicle (Feige et al., 2000) for control + veh and EtOH + veh rats. Again, all rats received subcutaneous injections of either 2.0 mg/kg sTNFR1 dissolved in PBS (EtOH + antagonists) or PBS alone (control + veh and EtOH + veh) at the time of surgery and every other day until sacrifice. Diet infusion was resumed at the normal rate no sooner than 2 h after recovery from anesthesia. Distraction began 6 days after fracture (6-day latency) and continued for 14 days at 0.2 mm b.i.d. (0.4 mm/day). Immediately after the 14-day distraction period, the animals were euthanized under anesthesia. Trunk blood was collected, allowed to clot on ice, centrifuged for 30 min at ~3000*g*, and serum was frozen at −20°C. Both the distracted and intact tibiae were collected and stored in 10% neutral-buffered formalin.

Measurement of Serum IL-1ra and sTNFR1. The concentrations of IL-1ra and sTNFR1 in trunk blood collected during the sacrifice of study 2 were determined using Quantikine human IL-1ra (R&D Systems, Minneapolis, MN) and Quantikine human sTNF R1 (R&D Systems) enzyme-linked immunosorbent assay according to the manufacturer's instructions.

Histology. After at least 48 h of storage in formalin, the distracted tibiae were removed from the fixators using a manual saw as described previously (Aronson et al., 2001). The specimens were decalcified in 5% formic acid, embedded in paraffin, cut, mounted, and stained with hematoxylin and eosin for histological analyses as described previously (Skinner et al., 1997). The sections chosen for analysis were selected to represent a central or near central gap location. This was accomplished by choosing slides that contained all four full thickness cortices with intact marrow spaces at both the proximal and distal ends. All procedures for quantitation of new bone formation in the DO gap were performed as described previously (Aronson et al., 2001). Briefly, the slides were video recorded and analyzed using NIH Image Analysis 1.49 (National Institutes of Health, Bethesda, MD) under low-power (1.25× objective) microscopic magnification. The relative areas of the distraction gap (bounded by the four cortices) and new bone within the gap (osteoid

matrix and including areas where new bone had been resorbed) were quantified, and the percentage of new bone formation was calculated by dividing the new bone area by the total gap area. The new bone area was measured by outlining the area of new bone matrix in the gap from a line connecting the cortices on the proximal (or distal) side of the fracture to the leading edge of the new bone. This is a measure of the total bone formation that took place over the 14-day distraction period and is not influenced by osteoclast activity for three reasons. First, osteoclasts are not present when the new osteoid is laid down nor when it is mineralized. Second, osteoclasts begin to appear in the adjacent host marrow space ~7 days after bone formation has begun. Third, the delay between formation and resorption places the osteoclasts ≥1 mm from the leading edge of bone being formed in the primary matrix front. Thus, the resorption area is included in the "new bone area" because new bone must have been formed in that area during the distraction period before the osteoclastic activity.

Peripheral Quantitative Computerized Tomography (pQCT) Analysis. To assess the ability of the antagonists to protect intact bone from ethanol-induced osteopenia, the nonfractured contralateral tibiae collected from study 2 were scanned by pQCT (XCT research SA; Norland, Fort Atkins, WI). Using the manufacturer's software version 5.40, three 0.26-mm-thick cross sections of each tibia were taken at 3, 4, and 5 mm distal to the proximal end with a voxel size of 0.10 mm. A threshold of 570 mg/cm^3 was used to distinguish cortical bone, and a threshold of 214 mg/cm^3 was used to distinguish cancellous bone throughout the experiment. The total volumetric content, density, and area of cortical, subcortical, trabecular, and total bone were determined for each slice (Ke et al., 2001). Using these threshold settings, it was determined that the ex vivo precision of volumetric content, density, and area of total bone, trabecular, and cortical regions ranged from 0.99 to 3.48% with repositioning.

Statistical Methods. Data obtained from both studies demonstrated normality and equal variance. The results of study 1 were analyzed using Student's t test. Analysis of both histological and pQCT data from study 2 was performed by one-way analysis of variance using Tukey's post hoc test. Results are presented as mean ± S.E.M. and were considered statistically significant if $p < 0.05$.

Results

All groups gained weight at an equivalent and steady rate throughout the respective study period. In the rats fed a diet containing ethanol, the UECs varied with the established pulsatile pattern from 100 to 550 mg/dl (average 220 mg/dl) as reported previously (Badger et al., 1993). Serum levels of IL-1ra and sTNFR1 in study 2 averaged 1195.8 ± 213.41 and 58.4 ± 9.05 ng/ml, respectively, for rats in the EtOH + antagonist group and were undetectable in control + veh and EtOH + veh rats.

Histology. In study 1, a comparison of histological sections from distracted tibiae revealed a significant increase in bone column formation in the EtOH + antagonist group (42 ± 8.1%) compared with the EtOH + veh group (11 ± 7.6%) ($p < 0.033$; Fig. 1A). In study 2, a comparison of distracted tibial histological sections revealed 1) a significant decrease in bone column formation in EtOH + veh (39 ± 3.5%) versus control + veh (58 ± 2.7%) ($p < 0.001$) (Brown et al., 2002), and 2) a significant increase in bone column formation in the EtOH + antagonist (53 ± 2.9%) versus EtOH + veh (39 ± 3.5%) ($p < 0.007$; Fig. 1B). Representative histological sections from study 2 are shown in Fig. 2.

pQCT. The volumetric BMD of the nonfractured intact contralateral tibiae from study 2 was significantly lower in both the EtOH + veh and EtOH + antagonist rats compared with control + veh (Fig. 3).

Discussion

A previous study, using the TEN model, demonstrated a dramatic ethanol-related impairment of bone formation during DO and a decrease in BMD of intact bone (Brown et al., 2002). The current study demonstrates that ethanol's inhibition of bone formation during DO is reproducible and that exogenous IL-1 and TNF antagonists allow regenerate bone formation in both male and female rats at levels comparable with those seen in control animals despite prior and continued exposure to ethanol. These results are consistent with recent studies that demonstrate the synergistic bone-sparing effects of IL-1 and TNF antagonists in other pathologies (Kimble et al., 1995; Feige et al., 2000). The lack of a treatment group that received IL-1 and TNF antagonists in a nonethanol setting renders it difficult to interpret the influ-

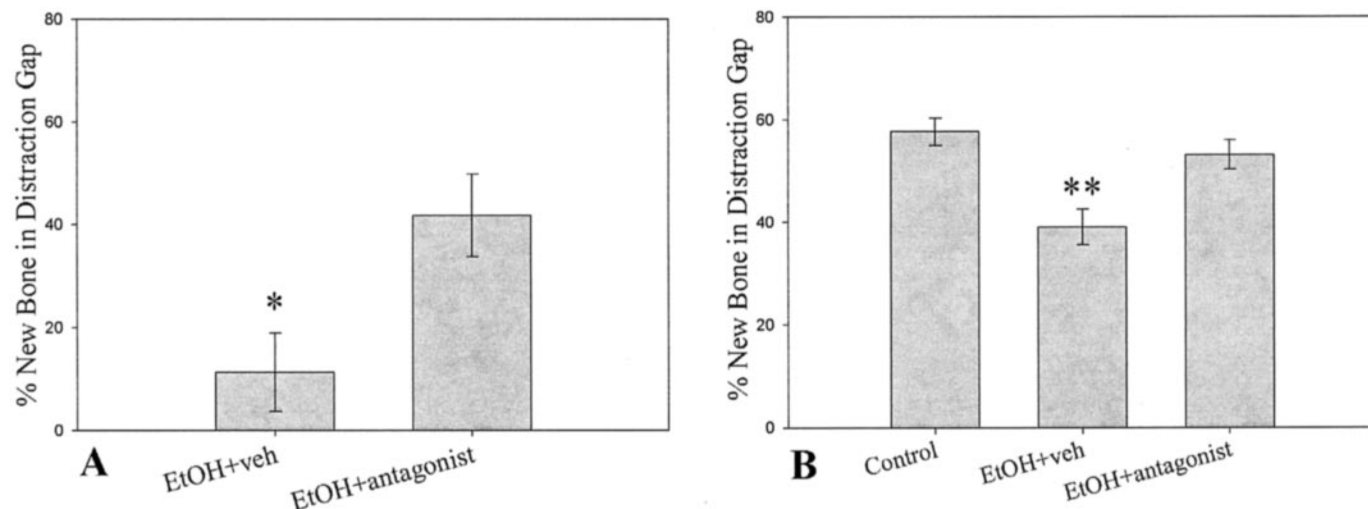

Fig. 1. Histological assessment of the percentage of the DO gap containing new bone after 14 days of distraction. IL-1 and TNF antagonists attenuate the effects of chronic EtOH on bone formation during DO. A, in study 1, histological analysis of distracted tibiae from female rats demonstrated a significant (*, $p < 0.001$) antagonist-related increase in the percentage of DO gap containing regenerate bone. B, histological analysis of distracted tibiae from study 2, conducted in male rats, shows a significant ethanol-related decrease in bone formation compared with control rats ($p < 0.001$) in addition to IL-1 and TNF antagonist-related protection of the regenerate ($p < 0.007$). Results are presented as mean ± S.E.M.

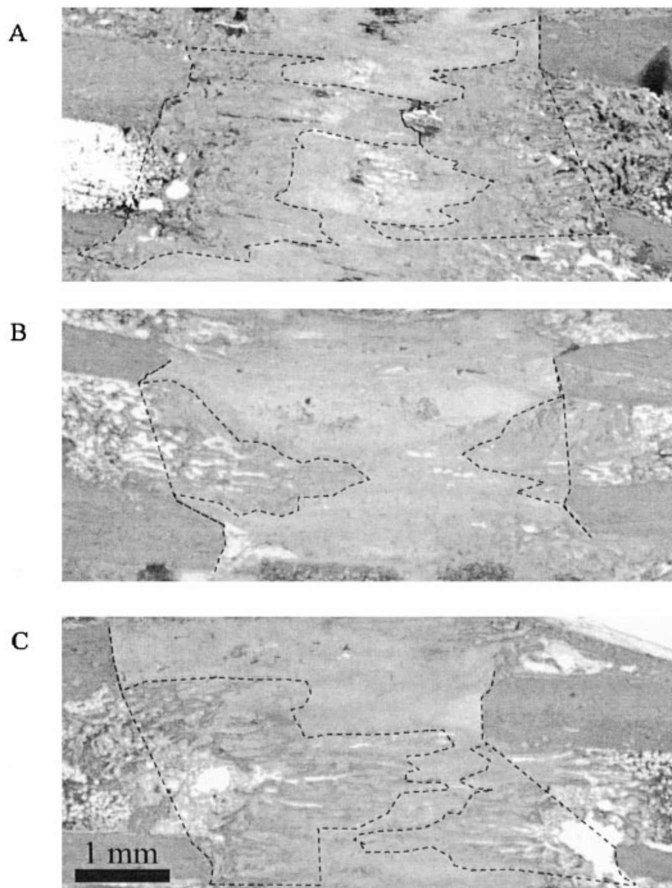

Fig. 2. Representative histological sections of distracted tibiae from control (A), EtOH + veh (B), and EtOH + antagonist (C) rats in study 2. The dashed line approximately encompasses the area of endosteal new bone in each section. Note the dramatic reduction in the area of regenerate bone in the EtOH + veh specimen (B). Original magnification, 1.25×.

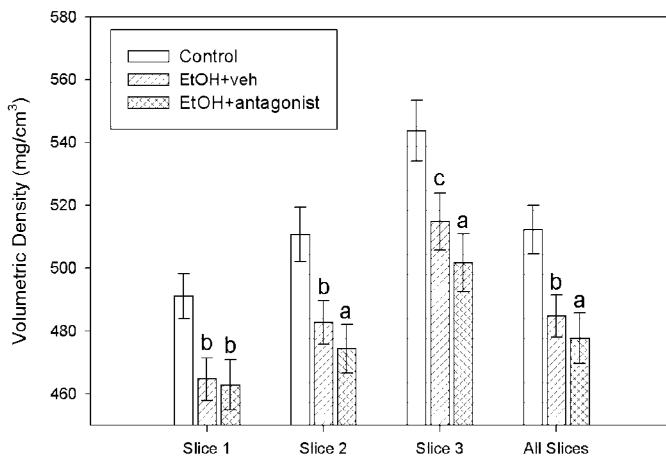

Fig. 3. Volumetric bone mineral density of intact contralateral tibiae as measured by pQCT. Treatment with chronic ethanol significantly reduced the volumetric BMD of the unfractured tibiaemetaphysis at 3, 4, and 5 mm distal to the epiphysis (slice 1, 2, and 3, respectively) in both EtOH + veh and EtOH + antagonist rats compared with control rats. These differences persist when the three slices are averaged (all slices). Results are presented as mean ± S.E.M. a, $p < 0.01$; b, $p < 0.05$; and c, $p < 0.10$.

ence that ethanol had on the ability of the antagonists to enhance bone formation during DO. However, the data clearly show that IL-1 and TNF antagonists attenuated ethanol-induced inhibition of bone formation during DO.

In contrast, these studies also indicate that the combined 20-day administration of IL-1 and TNF antagonists does not protect the volumetric BMD of intact (nonfractured) bone from ethanol-associated toxicity as measured by pQCT. This may be due to 1) the shorter duration of antagonist administration (20 days) relative to the ethanol exposure (31 days), and 2) alternative pathways through which ethanol may act on the intact skeleton but not repair/regenerative processes. Also, this may suggest that ethanol exerts its effects on the skeleton through multiple pathways that are tissue-type (regenerating versus mature)-dependent. At least one report by another group demonstrated a significant decrease in bone marrow levels of insulin-like growth factor-1 in response to acute ethanol administration (Turner et al., 1998). IL-6 is also thought to be a key mediator of ethanol-induced osteoclastogenesis and osteopenia in mice (Dai et al., 2000). Thus, these and other growth factors and/or cytokines may play a more prominent role in mediating the effects of ethanol on intact bone, whereas TNF or IL-1 may be more prominent mediators during repair and regeneration.

The results here suggest that high ethanol consumption results in local elevations of IL-1 and/or TNF activities that may inhibit osteoblastogenesis at multiple stages during bone repair. This is consistent with 1) studies demonstrating the ability of IL-1β and TNF-α to block multiple osteoblast functions in vitro as well as bone formation in vivo (Bertolini et al., 1986; Nguyen et al., 1991); 2) studies demonstrating that ethanol exposure increases the expression of IL-1 and TNF transcripts in the liver and bone marrow (Fang et al., 1998; Turner et al., 1998); 3) studies that demonstrate increases in TNF expression in the DO gaps of ethanol-exposed rats (D. S. Perrien, E. C. Brown, Z. Liu, R. A. Skinner, J. Aronson, L. J. Suva, T. M. Badger, and C. K. Lumpkin, manuscript submitted for publication); and 4) ongoing studies that demonstrate dramatic inhibitory effects of both recombinant TNF and IL-1 on the DO process in nonethanol-exposed rats (E. C. Brown, D. S. Perrien, L. Liu, J. Aronson, and C. K. Lumpkin, unpublished data). This DO/TEN model should facilitate the cellular and molecular studies necessary to elucidate the effects of alcohol on bone formation.

Currently, two general strategies are used to treat osteopenia: 1) an antiresorptive strategy that acts by inhibiting osteoclast activity, and 2) an anabolic approach that stimulates osteoblasts. Recently, a dual proresorptive/antiosteoblastic effect has been postulated for granulocyte colony-stimulating factor (Kuwabara et al., 2001). The results of this work suggest that IL-1 and/or TNF may function in a similar manner. Consequently, cytokine antagonists such as IL-1ra and sTNFR1 may provide such a dual protection of osseous repair and regeneration from the deleterious effects of chronic ethanol consumption.

Acknowledgments

We thank Matthew Ferguson, Shanda Ferguson, Kim Hale, and Brit Young for expert technical assistance.

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