IL-1 and TNF Antagonists Prevent Inhibition of Fracture Healing by Ethanol in Rats

Daniel S. Perrien,* †  ‡ Elizabeth C. Wahl,* William R. Hogue,* ‡ Ulrich Feige,‡ James Aronson,* † |||| Thomas M. Badger||| and Charles K. Lumpkin Jr.* †

*Laboratory for Limb Regeneration Research, Arkansas Children’s Hospital Research Institute, Little Rock, Arkansas 72202; †Department of Physiology and Biophysics, ‡The Center for Orthopaedic Research, College of Medicine, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205; §Department of Inflammation Research, Amgen, Inc., Thousand Oaks, California 91320; †Department of Pediatrics, ||Department of Toxicology, College of Medicine, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205; and ||||Arkansas Children’s Nutrition Center, Little Rock, Arkansas 72202

Received June 1, 2004; accepted September 22, 2004

We tested the hypothesis that combined administration of IL-1 and TNF antagonists would protect fracture healing from inhibition by chronic ethanol exposure. Adult male rats were fed a liquid diet ± ethanol (CON and ETOH) by intragastric infusion for three weeks prior to and three weeks after creation of an externally fixated tibial fracture. Beginning the day of fracture, one-half of each dietary group received 2.0 mg/kg/day IL-1ra and 2.0 mg/kg/2-days sTNFR1 (CON + ANTAG and ETOH + ANTAG), while all other animals received vehicle alone (CON + VEH and ETOH + VEH). Scoring of ex vivo radiographs and analysis by pQCT revealed a significantly lower incidence of bridging and reduced total mineral content in the ETOH + VEH group compared to all other groups. These results support, for the first time, the hypothesis that IL-1 and TNF antagonists are capable of protecting fracture healing from the inhibition associated with chronic ethanol consumption.

Key Words: alcohol; tumor necrosis factor; interleukin-1; interleukin-1 recombinant antagonist; repair; total enteral nutrition.

Chronic alcohol consumption is known to disrupt skeletal homeostasis and repair in multiple ways (Bikle, 1993; Brown et al., 2002; Chakkalakal et al., 2002; Purohit, 1997; Turner, 2000). Clinically, chronic alcoholics show a marked impairment in bone formation (Crilly et al., 1988) and a significant increase in their risk of non-union after fracture (Nyquist et al., 1997). Similar phenomena have been reported in rodents, including ethanol-related decreases in bone formation rate (Turner et al., 1987), osteoblast proliferation (Dyer et al., 1998), and delays in fracture healing (Chakkalakal et al., 2002; Elmali et al., 2002; Nyquist et al., 1999). In addition, at least one group has demonstrated ethanol-induced osteoclastogenesis in mice that appears to be dependent on induction of IL-6 (Dai et al., 2000).

Using the Total Enteral Nutrition (TEN) system of intragastric dietary delivery, this laboratory demonstrated that combined administration of IL-1 and TNF antagonists protected bone formation during distraction osteogenesis (DO) from ethanol-related inhibition (Perrien et al., 2002). It was later reported that the immunohistochemical expression of TNF-α but not IL-1β was significantly increased in the DO gap, and the mRNA levels of both cytokines were increased in the marrow of ethanol-fed rats (Perrien et al., 2003). Together, these studies provided the first evidence that one or both of these cytokines may play a role in the etiology of ethanol-related skeletal pathology.

Distraction osteogenesis is a variant of fracture healing in which an externally fixated fracture is literally stretched along the long axis of the bone in order to achieve limb lengthening. Obviously, there is a great deal of similarity between the cellular and molecular processes in both of these settings. This is especially true during the early inflammatory phases. However, once the lengthening (distraction) begins during DO, dramatic differences in tissue composition and behavior begin to take place. Most notably, the early cartilaginous/endochondral component of the fracture callus especially in the endosteal compartment is replaced by proliferative fibroblasts and osteoblasts as the mode of ossification becomes dominated by the intramembranous component.

The current study was designed to determine if the previous findings using the DO model are also applicable to the more commonly used rat model of fixated fracture. While these two models are similar, they do harbor several key differences that may have significant effects on the roles of numerous signaling pathways. Unfortunately, the differences in sensitivity to specific molecular signals during DO or fracture repair are poorly understood.

MATERIALS AND METHODS

Fifty virus-free adult male Sprague Dawley rats (three-months old, 350 g) 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. The rats were assigned to respective experimental groups with mean body weights equal to that of the control group (± 4 g) and weighed twice a week thereafter. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences and Arkansas Children’s Hospital Research Institute.

All surgical procedures for TEN have been described in detail elsewhere (Badger et al., 1993; Brown et al., 2002). All rats were handled by animal care personnel for 5–7 days prior to surgery. Briefly, a small silicone cannula was inserted through the wall of the stomach and tunneled subcutaneously to the head. It was then attached to a headpiece that was tethered to the top of the cage for infusion of the liquid diet. During the same surgery, stainless steel Illizarov-style ring fixators were applied to the left tibiae in standardized fashion (Aronson et al., 2001; Brown et al., 2002). All rats received 0.1 mg/kg buprenex for analgesia and were returned to their cages for observation during recovery from anesthesia. 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/kg0.75/day. Water was available ad libitum throughout the study. All rats were acclimated to the TEN system over a three-day period by gradually increasing the rate of dietary infusion to 187 kcal/kg0.75/day. Rats assigned to the ETOH groups were then acclimated to increasing amounts of ethanol (9–12 g/kg/day) in their diet over a one-week period and then maintained at 12 g/kg/day for the remaining five weeks of the study. For each calorie of ethanol added to the diet an isocaloric amount of carbohydrates was removed. Urine ethanol concentrations (UECs), which correlate positively with blood alcohol content, were measured daily for the duration of the study as previously described (Badger et al., 1993).

All rats assigned to the control groups were maintained on the control (non-alcoholic) diet for the duration of the experiment. Three weeks after placement of the intragastric cannula, all rats underwent a second surgery for creation of a transverse mid-diaphyseal osteotomy and implantation of an Alzet mini-osmotic pump (Durect, Cupertino, CA) for drug delivery as described in detail elsewhere (Perrien et al., 2002). At that time the CON and ETOH rats were further subdivided into two additional groups according to drug treatment: CON + VEH, CON + ANTAG, ETOH + VEH, ETOH + ANTAG. Under isoflurane anesthesia, three holes were drilled in the mid-diaphysis of the fixated left tibia. Direct lateral pressure was then applied to the weakened site to create the standardized fracture. For pump implantation, a 1 cm lateral subcutaneous incision was made on the back just above the scapulae. A small pocket was created using a blunt instrument, and an Alzet model 2ML2 osmotic pump delivering either 1.8 mg/kg/day IL-1ra (Amgen, Thousand Oaks, CA) (CON + ANTAG and ETOH + ANTAG) or vehicle (CON + VEH and ETOH + VEH) alone was inserted. After closing the incision, each animal received an im injection of 0.1 mg/kg buprenex for analgesia. In addition, rats in the antagonist treated groups received 2.0 mg/kg PEG-r-met-sTNFR1 (sTNFR1; Amgen) via sc injection at the time of surgery and every other day thereafter. Vehicle treated rats received equivalent sc injections of saline. Injections were given on alternating sides to minimize stress to the animals. After a 21-day healing period, the rats were euthanized under anesthesia. The operated tibiae were surgically removed by disarticulation at the knee and ankle and cleaned of all soft tissue prior to radiographic imaging and storage in 10% neutral buffered formalin.

**Peripheral quantitative computed tomography (pQCT) analysis.** The excised tibiae were scanned by pQCT (XCT research SA, Norland Medical Systems, Fort Atkins, WI) using the manufacturer’s software version 5.40 for slice reconstruction and analysis. A single cross-section of the central fracture callus between the proximal and distal host cortices was obtained with a voxel size of 110 μm. A threshold of 214 mg/cm3 was used to distinguish new bone from soft tissue within the callus. The total volumetric mineral content, density, and area of new bone were determined (Ke et al., 2001). Using these threshold settings, it was determined that the ex vivo precision of volumetric mineral content, density, and area of new bone ranged from 0.99 to 3.48% with repositioning.

**Micro-computed tomography (μCT).** Representative fracture calluses were selected based on standard radiographs and scanned in a μCT40 microCT unit (Scanco Medical, Bassersdorf, Switzerland). In order to visualize the entire callus at sufficiently high resolution, each specimen required acquisition of

FIG. 1. Peripheral quantitative computed tomography (pQCT) was used to measure volumetric mineral content (A), new bone volume (B), and volumetric density (C) in a single 110 μm-thick axial slice of the central callus at 21-days post-fracture. Analysis demonstrated a significant ethanol related reduction in volumetric mineral content (A) in the ETOH + VEH group compared to both the CON + VEH and CON + ANTAG group (p < 0.05). In contrast, none of the other three groups were found to be significantly different from one another, suggesting that treatment with IL-1 and TNF antagonists in the ETOH + ANTAG group was able to attenuate the ethanol-related inhibition of fracture healing. Analysis of new bone volume (B) and volumetric density (C) did not reveal any significant differences although a similar trend is seen in each of the data sets. All data was analyzed using a one-way ANOVA and SNK post hoc test.
700–900 cross sectional slices with a voxel size of 17 µm in all directions. The manufacturer’s software was then used to define a volume of interest on the two-dimensional slices. The slices were subsequently stacked into a three-dimensional cube before reapplying the VOI boundaries, an optimized grey scale threshold, and a Gaussian noise filter to create a binary three-dimensional rendering of the bony tissue.

Statistical methods. Incidence of radiographic union was compared by z-test of proportions. pQCT data was analyzed by one-way ANOVA and Tukey post hoc tests. Results were considered statistically significant when $p < 0.05$.

RESULTS

All groups gained weight at an equivalent and steady rate throughout the study period. The weights at sacrifice between the control and ethanol fed rats were not significantly different (control = 385 ± 6.7 g, ethanol = 370 ± 7.6 g). In the rats fed a diet containing ethanol, the UECs varied with the established pulsatile pattern from 100 to 550 mg/dl (average 206 mg/dl). Of the 56 rats that began the study 18 were sacrificed prior to completion of the protocol due to failure of the intragastric cannula, surgical complications, or other health problems. Consequently, these animals were excluded from all analyses reducing the final size of the groups to $n = 11$ for CON + VEH, $n = 10$ for CON + ANTAG, $n = 8$ for ETOH + VEH, and $n = 9$ for ETOH + ANTAG.

As a clinically relevant measure of repair, standard two-dimensional radiographs of the excised fracture calluses were qualitatively scored for presence or absence of radiographic union by a blinded, trained observer. Analysis revealed that, after 21 days of healing, fracture calluses from ETOH + VEH rats had a significantly lower incidence of radiographic union compared to all three other groups ($p < 0.05$; Table 1). The incidence of radiographic union was not significantly different between the remaining three groups.

Quantitatively, pQCT was used to measure the volumetric, mineral content, density, and volume of new bone in the central fracture callus, the same region scored for bridging. This analysis produced similar results to those found in the qualitative bridging analysis. Similar to the results of the bridging analysis, quantification of the mineral content showed the ETOH + VEH rats had significantly less mineral in the central callus than either CON + VEH or CON + ANTAG rats ($p < 0.05$ for both), which were not significantly different from each other. In contrast, the mineral content of ETOH + ANTAG rats was significantly lower than both CON + VEH and CON + ANTAG rats ($p < 0.05$ for both).

![FIG. 2. Representative three-dimensional microCT reconstructions of 21-day fracture calluses illustrate the complete bony union that is formed across the fracture site in the CON + VEH and CON + ANTAG specimens. In contrast, the ETOH + VEH specimen has failed to form any union while the ETOH + ANTAG specimen is almost completely bridged.](image-url)
not significantly different from any of the other three groups. While the differences in mineral content were the only measure
to reach statistical significance (p < 0.05; Fig. 1), a similar trend
was seen for both density and new bone volume. Again, this
demonstrates that, as expected, ethanol exposure impairs frac-
ture healing in vehicle treated rats, and treatment of ethanol fed
but not control fed rats with IL-1 and TNF antagonists can
enhance the process (Fig. 2).

DISCUSSION

Together, these results indicate that inclusion of ethanol in
the diet of vehicle treated rats impairs fracture repair by redu-
cing the likelihood of radiographic union and the mean vol-
metric mineral content at 21 days. More importantly, the
significantly higher incidence of union in ETOH + ANTAG
rats compared to ETOH + VEH treated rats and the lack of
a significant difference in mineral content between the ETOH +
ANTAG and CON + VEH groups suggests that antagonism of
IL-1 and TNF signaling was able to protect the repair process
from the inhibitory effects of ethanol exposure. In addition, the
apparent similarity between the CON + VEH and CON +
ANTAG groups also indicates that the impact of the antagonists
on fracture healing is specific to the condition of ethanol expo-
sure. This last conclusion is of particular importance as it
implies that IL-1 and/or TNF signaling may be of particular
importance in ethanol-related skeletal pathology and this is
supported by previously published data by this (Perrien et al.,
2002, 2003) and other laboratories (Turner et al., 1998).
Specifically, rats fed an ethanol containing diet using either
the TEN system or the Lieber-DeCarli paired feeding system,
have increased expression of TNF-α and/or IL-1β in femoral
marrow samples (Perrien et al., 2003; Turner et al., 1998). In
addition, this laboratory has documented increased expres-
sion of TNF-α in osteoblasts in response to ethanol exposure in vitro
and in vivo in ethanol fed rats undergoing distraction osteogen-
esis (Perrien et al., 2003). Thus, it is possible that, in the early
phase of fracture healing when the newly forming fracture gap
is contiguous with the bone marrow, excess ethanol induced
TNF in the marrow cells (stromal cells, T cells, etc.) negatively
affects bone formation. Once the fracture architecture is
formed, then direct ethanol induction in the fracture cells
(stromal cells, pre-osteoblasts, osteoblasts, chondroblasts, etc.)
of excess TNF is a possible source of osteoinhibition. Also,
since mature fractures are well vascularized a contribution of
mononuclear cells is still possible. Recent papers suggest
another level of complexity, that peripheral blood mononuclear
cells were shown to secrete TNF after co-culture with a stromal-
like cell line (Atkins et al., 2000; Nanes, 2003). Thus, cytokine
over production can also be modulated by cell-cell contact
and this possibility should be considered in this context. In
conclusion, the results of the current study are in line with
previously published data and add to a growing body of
evidence that induction of TNF-α and/or IL-1β is at least par-
tially responsible for ethanol-induced skeletal pathology.

ACKNOWLEDGMENTS

Supported in part by NIH grants AA12223 and AA08645, and NCRR grant
RR16517.

REFERENCES

and Lumpkin, C. K., Jr. (2001). The effect of aging on distraction osteogenesis
Atkins, G. J., Haynes, D. R., Geary, S. M., Loric, M., Crotti, T. N., and Findlay,
D. M. (2000). Coordinated cytokine expression by stromal and hematopoietic
Badger, T. M., Ronis, M. J., Lumpkin, C. K., Valentine, C. R., Shahare, M., Irby, D.,
Effects of chronic ethanol on growth hormone secretion and hepatic
cytochrome P450 isozymes of the rat. J. Pharmacol. Exp. Ther. 264,
438–447.
53–79.
Brown, E. C., Perrien, D. S., Fletcher, T. W., Irby, D. J., Aronson, J., Gao, G. G.,
and Lumpkin, C. K., Jr. (2002). Skeletal toxicity associated with chronic
ethanol exposure in a rat model using total enteral nutrition. J. Pharmacol.
Exp. Ther. 301, 1132–1138.
Chakalakal, D. A., Novak, J. R., Fritz, E. D., Mollner, T. J., McVicker,
Chronic ethanol consumption results in deficient bone repair in rats. Alcohol
Alcohol 37, 13–20.
Bone histomorphometry, bone mass, and related parameters in alcoholic
Dai, J., Lin, D., Zhang, J., Habib, P., Smith, P., Murtha, J., Fu, Z., Yao, Z., Qi, Y.,
and Keller, E. T. (2000). Chronic alcohol ingestion induces osteo-
estrogenesis and bone loss through IL-6 in mice. J. Clin. Invest. 106,
887–895.
inhibits osteoblast cell proliferation and activity in vivo. Alcohol 16,
337–341.
Elmali, N., Ertem, K., Ozen, S., Inan, M., Baysal, T., Guner, G., and Bora, A.
(2002). Fracture healing and bone mass in rats fed on liquid diet containing
Ke, H. Z., Qi, H., Chidsey-Frink, K. L., Crawford, D. T., and Thompson, D. D.
(2001). Lasofoxifene (CP-336,156) protects against the age-related changes in
bone mass, bone strength, and total serum cholesterol in intact aged male rats.
and healing of tibial shaft fractures in alcohol abusers. Alcohol Alcohol
32, 91–95.
Nyquist, F., Halvorsen, V., Madsen, J. E., Nordsletten, L., and Obrant, K. J.
Perrien, D. S., Brown, E. C., Fletcher, T. W., Irby, D. J., Aronson, J., Gao, G. G.,
and Lumpkin, C. K., Jr. (2003). Ethanol and its effects on fracture healing and
bone mass in male rats. Alcohol Alcohol 38, 91–95.


