Acute ethanol exposure impairs angiogenesis and the proliferative phase of wound healing

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Acute ethanol exposure impairs angiogenesis and the proliferative phase of wound healing. Am J Physiol Heart Circ Physiol 289: H1084–H1090, 2005. First published April 29, 2005; doi:10.1152/ajpheart.00080.2005.—Acute ethanol exposure represents an increased risk factor for morbidity and mortality associated with surgical or traumatic injury. Despite clinical observations suggesting that ethanol exposure before injury alters tissue repair processes, little direct evidence about the mechanism by which ethanol affects the wound healing process is available. In this study, excisional wounds from female BALB/c mice with or without circulating ethanol levels of 100 mg/dl were used to assess wound closure, angiogenesis, and collagen content. Ethanol exposure resulted in a significant but transient delay in wound closure at day 2 postwounding (28 ± 4% vs. 17 ± 1%). In addition, total collagen content was significantly reduced by up to 37% in wounds from ethanol-treated mice compared with controls. The most significant effect of ethanol exposure on wounds was on vascularity because angiogenesis was reduced by up to 61% in wounds from ethanol-treated mice. The reduction in vessel density occurred despite near-normal levels of proangiogenic factors VEGF and FGF-2, suggesting a direct effect of ethanol exposure on endothelial cell function. Further evidence for a direct effect was observed in an in vitro angiogenesis assay because the exposure of endothelial cells to ethanol reduced angiogenic responsiveness to just 8.33% of control in a cord-forming assay. These studies provide novel information regarding the effect of a single dose of ethanol on multiple parameters of the wound healing process in vivo and suggest a potential mechanism by which ethanol impairs healing after traumatic injury.

Wound healing normally proceeds without complications; nevertheless, tissue repair can be altered by many different factors, such as age and systemic disease. Interestingly, clinical impression suggests that alcohol intoxication at the time of injury may interfere with the restoration of damaged tissue. The importance of such an effect could be profound, because ethanol exposure is involved in over half of all emergency room trauma cases and has been implicated as an increased risk factor for morbidity and mortality in surgical and trauma patients (7, 9, 18, 23). Studies in a murine model system have demonstrated that ethanol exposure is associated with a reduced immune response after burn injury (11, 25). In addition, ethanol exposure has been shown to delay gastric reepithelialization in vivo and to impair collagen synthesis by fibroblasts in vitro (34, 38). To date, the effect of acute ethanol exposure on the wound healing process has received minimal experimental attention.

Most studies involving the effect of ethanol exposure on the response to injury have focused on chronic exposure. The response to acute versus chronic ethanol exposure can be variable depending on the pathological state of the tissue and cell type (20, 26, 29, 35). Because the frequency of acutely intoxicated trauma victims is as high as for those who present with chronic ethanol exposure, the mechanistic changes in tissue repair induced by acute ethanol exposure must also be elucidated to develop interventions that promote optimal wound healing.

On the basis of the clinical observation that acute ethanol exposure increases morbidity and mortality after surgical or traumatic injury, we hypothesized that acute exposure to alcohol before injury may impair the wound healing process itself. The effects of ethanol on the proliferative phase of dermal wound healing were examined by assessing wound closure, collagen production, and angiogenesis. Surprisingly, our studies not only demonstrate that a single exposure to ethanol 30 min before injury has long-term effects on the ability to repair damaged tissue but also suggest that the mechanism may include interference with the cellular response to stimulatory factors.

MATERIALS AND METHODS

Administration of alcohol and punch wounds. Eight- to nine-week-old female BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) weighing between 17 and 21 g were administered a 150-μl ip...
injection of a 20% ethanol solution or 150 μl of saline as a control. After 30 min, the ethanol-treated mice had a circulating blood alcohol concentration of 100 mg/dl, which is equivalent to 0.1% blood alcohol concentration and is just above the legal limit in most states (25). The mice were subsequently anesthetized with pentobarbital sodium (Nembutal, 50 mg/kg ip; Abbott Laboratories, North Chicago, IL) according to their body weight. When completely anesthetized, each mouse had its dorsum shaved, and six full-thickness excisional punch wounds were placed with the use of a 3-mm biopsy punch (Acu Punch; Acuderm, Fort Lauderdale, FL). After 8 h, the mice had a circulating blood alcohol concentration of ~23 mg/dl, and by 24 h the ethanol was undetectable. At specific time points after injury, mice were killed and individual wounds were harvested from the pelt with the use of a 5-mm punch biopsy instrument. In each hydroxyproline, FGF-2, and VEGF analysis, the level was normalized to wet weight by dividing by the average wet weight (mg) as determined for each time point. For each of the different excisional wound analyses, one of the six wounds was randomly selected from each mouse, and this single wound was utilized for that unique analysis. Because the mice appeared to behave normally after surgery, and because anesthetics might interfere with wound healing outcomes, no anesthetics were administered. Antibiotics were not found to be needed because post-operative infection is exceptionally rare in this model system. Animal protocols used in these studies were reviewed and approved by the Loyola University Institutional Animal Care and Use Committee.

Scald burn wound. The dorsal scald injury was produced as previously described (25). After induction of anesthesia (Nembutal, 50 mg/kg ip), mice were shaved to expose the dorsal surface. Mice were placed in a plastic template with a 12-cm² window that exposed ~15% of their total body surface area (33). The mouse, held in the template, was then immersed in a 100°C water bath for 8 s. After this, mice were dried with a towel, resuscitated with 1.5 ml 0.9% saline ip, and placed under warming lamps until recovery from anesthesia. At day 10 after injury, wound tissue was obtained and embedded in OCT for analysis of angiogenesis as described in Analysis of wound angiogenesis. This time point was selected after preliminary studies in our laboratory suggested that day 10 is a period of robust vascular growth in the scald wound model (Ref. 10; DiPietro LA, unpublished observation).

Analysis of wound reepithelialization. Wound reepithelialization of excisional wounds (n = 4 mice per group, one wound per mouse) was measured using histomorphometric analysis of 10-μm tissue sections from the middle of the wound as described previously (30). Briefly, sections were stained with hematoxylin and eosin. The percentage of reepithelialization [(distance covered by epithelium/distance between wound edges) × 100] was calculated for each section and performed blinded. The average reepithelialization between two wound sections for each animal was used as a unique value.

Analysis of wound collagen content. The hydroxyproline content of single individual wounds (n = 4–6 mice per group, one wound per mouse) was determined according to a standard protocol (39). Each wound was harvested from the pelt with the use of a uniform 5-mm biopsy punch. Because the specimens contain some adjacent normal skin, this analysis compares relative differences between treatment groups rather than absolute values. Because the amount of normal skin is nearly identical in all samples, any observed differences were expected to reflect that of the wound and not normal skin. All reagents for this assay were purchased from Sigma Chemical (St. Louis, MO). Briefly, frozen tissue was hydrolyzed in 2 ml of 6N HCl overnight at 110°C. The reaction was neutralized with 2.5N NaOH and diluted 40-fold with MilliQ water. One milliliter of a 0.05 M chloramine-T solution was added to 2 ml of the neutralized and diluted solution and incubated for 20 min at room temperature. One milliliter of 3.15 M perchloric acid was added, and the solution was incubated for 5 min at room temperature. One milliliter of 20% p-dimethylaminobenzaldehyde was subsequently added, and the mixture was incubated for 20 min at room temperature. The samples were then cooled with cold tap water. The amount of hydroxyproline was determined by comparison to a standard curve measured spectrophotometrically at an absorbance of 557 nm. Samples were analyzed in duplicate.

Analysis of wound angiogenesis. Wound vessel density (n = 3–4 mice per group, one wound per mouse) was determined after immunohistochemical staining with an antibody specific to platelet-derived endothelial cell adhesion molecule-1 (PECAM-1 or CD-31; Phar-Mingen, San Diego, CA), a marker for vascular endothelial cells, as described previously (22). Briefly, 10-μm sections were fixed in acetone for 15 min at room temperature, pretreated with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity, and then blocked for nonspecific binding with 50 μl normal mouse serum (1:100; Sigma). Secondary antibody (biotinylated-mouse-α-Rat IgG; Jackson, West Grove, PA) incubations were performed for 30 min each. Sections were subsequently incubated for 30 min with avidin-biotin horseradish peroxidase complexes (ABC Vectastain; Vector Laboratories, Burlingame, CA). Color development was performed with 3,3′-diaminobenzidine (Kirkegard and Perry, Gaithersburg, MD), and sections were counterstained with Harris hematoxylin. Image analysis software was utilized to determine vessel staining (Scion Image Software, Optronics Engineering Software). The PECAM-positive area within the wound bed was assessed, and the percent vascularity was calculated as (PECAM-positive area/total wound bed area) × 100%. The average percent vascularity (vessel density) between two wound sections for each animal was used as a unique value.

Analysis of wound FGF-2 and VEGF levels. Dermal FGF-2 and VEGF levels were determined with the use of a human FGF-2 ELISA kit or murine VEGF164 (mVEGF164) ELISA kit (Quantikine; R&D Systems, Minneapolis, MN) as described previously (36). The amount of growth factor in unique individual wounds (one from each mouse) was determined and normalized to the average wet weight of wounds for each specific time point. Because both FGF-2 and VEGF are nearly undetectable in normal skin, any adjacent normal tissue that was included in the sample added only negligible amounts to the total measured value. To perform this analysis, individual wounds (n = 3–4 mice per group, one wound per mouse) were removed from the pelt with a 5-mm biopsy punch and were placed in 1 ml of homogenization buffer [one protease inhibitor tablet (Boehringer-Mannheim) in 50 ml Dulbecco’s PBS (D-PBS, Gibco-BRL)]. Wounds were homogenized at high speed for 10–15 s. The homogenate was then sonicated at 30% power for 10 s. Samples were then centrifuged at 800 g for 2 min at 4°C, transferred to 1.5-ml centrifuge tubes, and centrifuged at 2,000 g for 10 min at 4°C. The supernatant was then filtered through a 1.2-μm pore syringe filter and snap frozen in liquid nitrogen. Samples were analyzed in duplicate. Both ELISA assays were performed according to the manufacturer’s protocol. Wound VEGF and FGF-2 levels were derived from a standard curve read at an absorbance of 450 nm in a spectrophotometer.

In vitro cord formation assay. Murine Simian Virus 40-transformed murine small vessel lymph node endothelial cell lines (SVEC4–10, American Type Culture Collection, Rockville, MD) at a concentration of 5 × 10⁴ ml were stimulated for 30 min at room temperature with DMEM-10% FBS only; DMEM-10% FBS and 100 ng/ml of mVEGF164 (R&D Systems); DMEM-10% FBS, 100 ng/ml of mVEGF164, and 100 mg/dl of ethanol; or 100 mg/dl of ethanol and DMEM-10% FBS. After incubation, 1 ml of the cell suspension was subsequently plated onto 35-mm culture dishes coated with 1 ml of collagen gel containing 4 mg/ml of rat tail collagen (Upstate Biotechnology, Lake Placid, NY) in PBS, pH 7.4. After incubation for 4 h at 4°C with 5% CO₂ on the collagen gel, six random fields were chosen on the culture dish and were photographed. Cordlike structures were counted per field, and the average number of cordlike structures per field was calculated for control and experimental groups. Cord formation is judged as three or more endothelial cells involved in an adherence network formation. For each independent experiment, the number of endothelial cordlike structures formed in the presence of
control murine VEGF164 was considered maximal (100%), and experimental values were determined as a percentage of maximal cord formation. This experiment was performed in quadruplicate. The mean cord formation for each group was subjected to statistical analysis using one-way ANOVA followed by Bonferroni’s posttest.

Statistical analysis. Data were analyzed using GraphPad Prism (version 2.1, GraphPad Software, San Diego, CA). The values were calculated as means ± SE for each data set. For analysis of excisional wound reepithelialization, collagen content, angiogenesis, FGF-2, and VEGF, data were analyzed by a two-way ANOVA and either Student’s t-test or Bonferroni’s posttest. Burn wound angiogenesis was analyzed by an unpaired t-test. A one-way ANOVA and Bonferroni’s posttest were used for analysis of cord formation. P values <0.05 were considered significant.

RESULTS

Acute ethanol exposure delays wound reepithelialization. An integral part of the wound healing process involves the regeneration of the epithelium to reestablish a barrier from external pathogens. To determine whether acute ethanol exposure influenced wound reepithelialization, we examined epidermal coverage of the wound bed. At day 2, wound sections obtained from ethanol-treated mice were less reepithelialized (Fig. 1) compared with those of saline controls (17.3 ± 1.2% vs. 30.3 ± 3%). However, by day 3 there was no significant difference in wound coverage between the two treatment groups, with wounds from both treatment groups completely reepithelialized by day 5 (by day 3: 34.0 ± 8.0% ethanol vs. 41.5 ± 4.3% saline; by day 5: 97.8 ± 2.1% ethanol vs. 100 ± 0% saline). These data suggest that the acute ethanol exposure delays but does not inhibit keratinocyte migration across the wound bed.

Acute ethanol exposure decreases wound collagen content. Restoration of the extracellular matrix is necessary to reestablish the strength and flexibility of the injured dermis. To determine whether acute ethanol exposure would alter collagen content in the wound, we quantified the total levels of collagen in wounds from saline- or ethanol-treated mice (Fig. 2). Hydroxyproline, used as an index for total collagen content, was significantly reduced at day 7 postwounding in wounds from ethanol-treated animals compared with saline controls (5.94 ± 1.56 vs. 9.43 ± 0.56 μg/mg wet wt). These results are consistent with previous in vitro observations in which ethanol exposure inhibited collagen synthesis by fibroblasts and increased levels of matrix-degrading proteins (4, 21, 34). Although these data do not reveal the mechanism, it is evident that acute ethanol exposure significantly impairs the regeneration of the dermal extracellular matrix.

Acute ethanol exposure inhibits wound angiogenesis. During wound healing, restoration of the extracellular matrix and angiogenesis occur concurrently. Physiological angiogenesis is contingent on the interaction of endothelial cells with the extracellular matrix. Because we observed a significant reduction in wound collagen content, we sought to determine whether acute ethanol exposure would also impair wound angiogenesis. Photographs of wounds from ethanol-treated mice exhibited fewer vessels compared with wounds from saline-treated mice (Fig. 3A). Vasculature from wounds of saline-treated mice appears to migrate toward the epithelium. Vasculature from wounds of ethanol-treated mice is reduced and lacks directional migration toward the epithelium. Analysis of vessel density (Fig. 3B) revealed significantly reduced wound vessel density at days 7, 10, and 14 postwounding in wounds from ethanol-treated animals compared with saline controls (day 7: 6.0 ± 0.8% vs. 10.1 ± 1.2%; day 10: 4.4 ± 1.1% vs. 9.4 ± 0.4%; day 14: 3.8 ± 0.4% vs. 9.7 ± 0.5%). These results demonstrate that acute ethanol exposure has a negative influence on wound angiogenesis.

To determine whether the observed effect of ethanol exposure on wound angiogenesis was specific to wound type, we examined how acute ethanol exposure affected wound vascularity in a burn wound model. This analysis showed that, similar to excisional wounds, a single ethanol exposure caused a significant reduction in the revascularization of scald burn wounds (Fig. 3C).

Acute ethanol exposure alters wound FGF-2 and VEGF content. To further assess the effect of acute ethanol exposure on wound angiogenesis, we examined the levels of two key components of the angiogenic pathway. During the process of angiogenesis, FGF-2 and VEGF are involved in vessel formation. We found that acute ethanol exposure significantly reduced the levels of FGF-2 in wounds from ethanol-treated mice compared with saline controls (day 3: 315.6 ± 56.0 vs. 631.5 ± 56.0 pg/mg wet wt; day 7: 100.2 ± 25.2 vs. 200.3 ± 25.2 pg/mg wet wt; day 10: 25.2 ± 5.6 vs. 50.4 ± 5.6 pg/mg wet wt; day 14: 25.2 ± 5.6 vs. 50.4 ± 5.6 pg/mg wet wt). Similarly, the levels of VEGF were also significantly reduced in wounds from ethanol-treated mice compared with saline controls (day 3: 1475.6 ± 256.0 vs. 2951.5 ± 256.0 pg/mg wet wt; day 7: 737.5 ± 125.2 vs. 1475.6 ± 125.2 pg/mg wet wt; day 10: 125.2 ± 25.6 vs. 250.4 ± 25.6 pg/mg wet wt; day 14: 125.2 ± 25.6 vs. 250.4 ± 25.6 pg/mg wet wt). These results indicate that acute ethanol exposure inhibits the expression of FGF-2 and VEGF, which are key regulators of angiogenesis.

Fig. 1. Acute ethanol exposure delays wound reepithelialization. Percentage of reepithelialization [(distance covered by epithelium/distance between wound edges) × 100] was calculated at days 2, 3, and 5 postwounding. Reepithelialization of wounds from ethanol-treated mice (circles) was significantly delayed compared with wounds from saline-treated mice (squares). Percent reepithelialization data are means ± SE; n = 4 mice per group. *P < 0.05 by Student’s t-test.

Fig. 2. Acute ethanol exposure reduces wound collagen content. Wounds from ethanol- and saline-treated mice from day 2 to day 21 were collected and hydrolyzed. Hydroxyproline (HP) content, used as an index for the presence of collagen, was determined in wounds from ethanol-treated (circles) or saline-treated (squares) mice using a standard biochemical assay. NS, normal skin. HP content data are means ± SE for each day; n = 4 for saline-treated mice; n = 6 for ethanol-treated mice. *P < 0.01 for day 7 by two-way ANOVA and Bonferroni’s posttest.
proangiogenic cytokines in wounds. Both FGF-2 and VEGF are potent inducers of both physiological and pathological angiogenesis, with VEGF being the most critical cytokine for wound angiogenesis (3, 6, 12, 13, 22, 27, 28, 37). When compared with wounds from saline-treated mice, the level of FGF-2 was lower in wounds from ethanol-treated mice at only a single time point of day 7 (6.59 ± 1.32 vs. 13.05 ± 1.96 pg/mg wet weight) (Fig. 4A). In contrast, levels of VEGF in wounds from ethanol-treated mice never declined below the levels found in wounds from saline-treated mice (Fig. 4B). At day 10, VEGF levels were significantly higher in wounds from ethanol-treated mice compared with those from saline-treated mice (10.13 ± 2.53 vs. 0.66 ± 0.52 pg/mg wet wt). Overall, Fig. 3. Acute ethanol exposure reduces wound vessel density. A: histology of day 7 wound sections from ethanol-treated mice. Frozen sections from day 5 to day 21 were stained with anti-platelet-derived endothelial cell adhesion molecule-1 antibodies. Area of the wound bed that was used for vascular density analysis is indicated by the dashed line. Percent vascularity for each section is depicted in bottom left corner. B: percent vascularity (vessel density) was calculated for wounds in ethanol-treated (circles) or saline-treated (squares) mice by using image analysis software (Scion Image). Percent vascularity data are means ± SE for each day; n = 3 for saline- and ethanol-treated mice at 24 h; n = 4 for saline- and ethanol-treated mice at all other time points. *P < 0.05 for day 7 by two-way ANOVA and Bonferroni’s posttest. Analysis was performed in duplicate. C: percent vascularity (vessel density) was calculated for dorsal scald wounds of ethanol-treated or saline-treated mice by using image analysis software (Scion Image) at day 10 after injury. Percent vascularity data are means ± SE; n = 4 mice per group. *P < 0.002 by unpaired t-test.

Fig. 4. Acute ethanol exposure alters peak levels of FGF-2 (A) and VEGF (B) in wounds. Wounds from day 1 to day 21 postwounding were collected and homogenized. A: FGF-2 levels in wound homogenates from ethanol-treated (circles) and saline-treated (squares) mice were determined by ELISA. Peak of FGF-2 expression occurred at day 5 in wounds from ethanol-treated mice compared with day 7 in wounds from saline-treated mice. FGF-2 data are means ± SE for each day; n = 3 for saline- and ethanol-treated mice at 24 h; n = 4 for saline- and ethanol-treated mice at all other time points. *P < 0.05 for day 7 by two-way ANOVA and Bonferroni’s posttest. Analysis was performed in duplicate. B: VEGF levels in wound homogenates from ethanol-treated (circles) and saline-treated (squares) mice were determined by ELISA. VEGF data are means ± SE for each day; n = 4 for saline-treated mice at all time points; n = 6 for ethanol-treated mice at all time points. *P < 0.01 for day 10 by two-way ANOVA and Bonferroni’s posttest.
Acute ethanol exposure induced a modest decrease in FGF-2 levels but prolonged VEGF production in wounds.

Acute ethanol exposure inhibits cord formation in vitro. Because we observed a significant reduction in wound vessel density despite prolonged VEGF production, we questioned whether acute ethanol exposure might directly influence endothelial cell responsiveness to growth factors. To examine this possibility, we employed a well-characterized in vitro cord formation assay in which endothelial cells form cordlike structures in the presence of VEGF. Exposure to 100 mg/dl of ethanol caused a significant inhibition of the formation of cordlike structures in response to VEGF (Fig. 5A). The level of cord formation by cells exposed to VEGF and ethanol averaged just 8.33% of that of control cells exposed to VEGF alone (Fig. 5B). To determine whether the endothelial cells could recover from the effects of ethanol exposure, we incubated the cells for 4 h with or without ethanol at 100 mg/dl, removed the ethanol-containing media, and then allowed the cells to recover for 8 h in ethanol-free media. The cells were then plated on the collagen layer with VEGF and allowed to incubate for 4 h. The ability of the endothelial cells to differentiate into cordlike structures was significantly inhibited by preexposure to ethanol. The number of cords formed in the ethanol preexposed cultures reached just 37.3 ± 3.7% of control values. Together, the data indicate that acute ethanol exposure can directly alter the endothelial cell response to differentiation signals in vitro and that this effect may be long lasting. Furthermore, these studies provide an in vitro correlate of our in vivo observation of reduced angiogenesis in wounds from ethanol-treated animals.

**DISCUSSION**

Although our understanding of normal wound repair mechanisms has dramatically advanced in recent years, studies of abnormal wound healing have provided an increasingly complex picture. Both exogenous as well as pathological factors have been demonstrated to induce distinct cellular changes that result in abnormal wound healing (26, 36, 40). As new factors emerge as potential mediators of altered wound repair, the necessity for novel interventions to promote optimal healing becomes increasingly apparent. Despite the clinical evidence that ethanol exposure before surgical or traumatic injury increases the risk for morbidity and mortality, the effects of ethanol on wound repair have yet to be fully elucidated. Our studies demonstrate that a single ethanol exposure equivalent to moderate intoxication (100 mg/dl) can dramatically impair the proliferative phase of dermal wound healing. Wound reepithelialization was moderately delayed in wounds from ethanol-treated mice, consistent with a previous study that demonstrated delayed gastric reepithelialization using a topical application of 50% ethanol (38). The significant reduction in collagen synthesis in wounds from ethanol-treated mice correlates well with previous studies in other systems that associate ethanol exposure with both decreased collagen synthesis and elevated levels of matrix-degrading proteins (15, 21, 34). Moreover, the tensile strength of wounds from intoxicated patients appears to be compromised because an increased incidence of intestinal wound dehiscence has been observed in these patients (32). The magnitude of variation in collagen content and structure may go well beyond connective tissue strength itself. The extracellular matrix not only functions as a reservoir for growth factors and signaling molecules but also acts as a framework on which endothelial cells can migrate during angiogenesis (6, 19).

The most remarkable impairment after acute ethanol exposure was the significant inhibition of wound angiogenesis. Cytokines produced during the inflammatory phase of wound healing promote initiation of the proliferative phase, resulting in reepithelialization of the wound bed, restoration of the extracellular matrix, and angiogenesis. Growth factors released by the migrating epithelium signal fibroblasts and endothelial cells to initiate collagen synthesis and angiogenesis, respec-
tively. Wound angiogenesis is contingent on chemokine secretion, extracellular matrix interactions, and equilibrium between proangiogenic and antiangiogenic factors (17, 27, 28). Migration of endothelial cells into the extracellular matrix is dependent on these cell-cell and cell-matrix interactions. The reduction in wound vascularity could result from a decrease in proangiogenic stimuli, a decrease in endothelial cell response, or a combination of these two events. With regard to the available proangiogenic stimulus, the wounds from ethanol-treated mice exhibited lower levels of FGF-2 at a single time point; however, the wounds contained normal or above normal levels of VEGF at all time points examined. Although it is possible that the reduction in FGF-2 played a role in the observed reduction in vessel density, this seems unlikely to be the sole mechanism of angiogenic impairment for several reasons. First, the angiogenic response of ethanol-treated mice was decreased even at the early time point of day 5, a time when both FGF-2 and VEGF levels were similar to or greater than saline-treated mice. Second, although both FGF-2 and VEGF are characterized as proangiogenic cytokines, previous studies show that VEGF is probably the more critical of the two in wound angiogenesis (22, 28, 37). Although peak VEGF levels in wounds from ethanol-treated mice were consistently equal to or greater than in wounds from saline-treated mice, wound vessel density in ethanol-treated mice was generally less than half that of saline-treated mice. Thus it seems likely that the effect of ethanol on wound angiogenesis is more complex than a simple reduction in the proangiogenic stimulus. Interestingly, ethanol exposure has also been shown to increase both FGF-2 and VEGF levels in exercised skeletal muscle (14).

Our in vitro results from the endothelial cord formation assay demonstrate that ethanol can directly alter the endothelial cell response to proangiogenic stimuli. Together, our in vitro and in vivo data suggest that the effect of ethanol on the angiogenic process probably involves impairment of endothelial cell responsiveness to proangiogenic stimuli. Previous studies in several cell types suggest that ethanol can directly affect cell function and may modulate PKC activity, membrane lipid composition, and receptor regulation (2, 16, 24, 35). Although the mechanism by which ethanol inhibits the endothelial cell response to a proangiogenic signal is not yet known, one likely possibility is that ethanol exposure inhibits VEGF receptor expression or function. Because endothelial proliferation, migration, and differentiation are primarily regulated by VEGF signaling, any change in receptor responsiveness would directly diminish wound angiogenesis. The mechanism by which ethanol might exert such an effect on endothelium could include VEGF receptor downregulation and/or a change in VEGF receptor phosphorylation in response to ligand binding. One additional possibility is that changes in membrane fluidity might also alter signal transduction by preventing dimerization of VEGF receptors and, hence, intracellular proangiogenic signaling. Our in vitro data support the notion that a single dose of ethanol can exert significant alterations in the endothelial response to VEGF that may be more than transient because the effect of ethanol exposure is maintained for a minimum of 8 h. The specific mechanistic changes in endothelial VEGF cell signaling induced by ethanol exposure, as well as the ability of ethanol to interfere with endothelial cell proliferation, remain to be investigated.

Our findings provide new evidence demonstrating that ethanol exposure can influence multiple aspects of wound healing. Importantly, the effect of ethanol on wound angiogenesis appears to occur independent of wound type because burn wounds exhibited an ethanol-mediated reduction in vascularity that was similar to excisional wounds. Together, these data suggest that the effect of a single exposure to ethanol on wound healing traverses several wound types. At this time, the precise mechanism by which ethanol and its metabolites induce these changes remains undefined. The most intriguing observation is that the effects of acute ethanol exposure on healing can be detected days after the ethanol has been cleared from the circulation. The long-lasting effect of a single ethanol exposure suggests that early perturbations may have prolonged effects. One possibility is that early effects have enduring consequences for healing. The known interconnections between the three phases of wound healing support this possibility. If ethanol influences wound inflammation, this might culminate in delayed wound closure. Alterations in the early inflammatory phase have been shown to profoundly affect the proliferative response in healing wounds (8), and previous studies suggest that acute ethanol exposure can alter the local inflammatory response (10, 25). In addition to inflammatory changes, acute ethanol exposure may mediate its effects directly on those cells involved in the proliferative phase of wound healing. In support of the idea that ethanol inhibits endothelial cell function directly, acute ethanol exposure has recently been shown to inhibit endothelial cell activation in vivo in a model of acute inflammation (31). If several days are required for endothelial cell recovery, the direct consequences of acute ethanol exposure might reasonably include delayed capillary outgrowth in wounds.

Our studies provide the first description of the precise aspects of wound healing that are impaired by acute ethanol exposure. These results suggest that the increase in morbidity and mortality that occurs in trauma victims with ethanol exposure can be somewhat attributed to global alterations in the wound healing process. These findings also demonstrate a possible need for treatment modifications for patients who are acutely intoxicated at the time of traumatic injury. Additional studies are needed to elucidate the precise mechanistic cellular changes that are evoked by acute ethanol exposure.

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