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Inflammatory response in multiple organs in a mouse model of acute alcohol intoxication and burn injury*

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Abstract

The present study characterized the inflammatory response following burn injury and determined whether ethanol (EtOH) intoxication at the time of burn injury influences this response. To accomplish this, male mice were gavaged with EtOH (2.9 g/Kg) 4 hours prior to 12–15% total body surface area sham or burn injury. Mice were sacrificed on day one after injury; blood, small intestine, lung and liver were collected to measure IL-6, IL-10, IL-18 and MCP-1 levels. In addition, neutrophil infiltration, MPO activity and edema formation were also measured in the small intestine, lung and liver. There was no difference in the inflammatory markers in the small intestine, lung and liver in mice receiving either sham or burn injury alone except IL-6 which was increased in all 4 tissue compartments following burn injury alone. However, as compared to EtOH or burn injury alone, EtOH combined with burn injury resulted in a significant increase in cytokines, neutrophil infiltration, MPO activity and edema in the small intestine, liver and lung tissue. Furthermore, a significant increase in IL-6 and MCP-1 was observed in circulation following EtOH and burn injury compared to either EtOH intoxication or burn injury alone, no other cytokines were detected in circulation. These findings suggest that acute EtOH intoxication exacerbates the inflammatory response following burn injury.

Keywords

Thermal injury; ethanol; cytokines; chemokines; leukocytes; neutrophils; tissue damage

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Introduction

Alcohol remains the most abused substance in the United States and other parts of the world. An association between alcohol and trauma has long been recognized¹. Nearly, one million burn injuries are reported every year in the United States² and half of these are found to be under the influence of alcohol/ethanol (EtOH) intoxication^{3–7}. Alcohol exposure at the time of injury has also been shown to have adverse affect on the recovery of the injured patients^{4–6, 8}. The patients who are intoxicated at the time of injury require more surgical procedures, have a longer hospital stay and exhibit a delay in wound healing compared to the patients who did not consume alcohol prior to sustaining the injury^{3–8}.

Several lines of evidence indicate that burn injury regardless of prior alcohol exposure induces an inflammatory response characterized by uncontrolled production of inflammatory mediators, including cytokines, chemokines and leukocyte infiltration^{9–11}. Furthermore, experimental data indicate that EtOH intoxication at the time of burn injury exacerbates the suppression of host immune response as characterized by antigen presentation, T cell activation and phagocytic capability^{7, 12}. Such decrease in host defense may enhance susceptibility to bacterial infection^{3, 7, 13}. Although, the initial release of cytokines or other inflammatory mediators is a normal host response to injury, if remains uncontrolled, it can lead to multiple organ dysfunction and failure, which is a major cause of death in injured patients. Since alcohol abuse is also associated with tissue damage¹³, we attempted in the present study to characterize the inflammatory response in various organs following burn injury and determine whether EtOH intoxication at the time of burn injury has any influence on this.

Materials and methods

Animals and reagents

Male C57BL/6 mice (22–25g) were obtained from Harlan Laboratories. All the animal procedures were carried out in adherence to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. These studies were initiated at the University of Alabama at Birmingham (UAB) and were approved by UAB and the authors' current institution Loyola University Chicago Medical Center Institutional Animal Care and Use Committee. EtOH kit was obtained from Roche (Nutley, NJ). Mouse IL-6, MCP-1 and IL-10 ELISA kits were obtained from BD Biosciences (San Jose, CA) and mouse IL-18 ELISA kit was obtained from Bender MedSystems (Burlingame, CA).

Blood EtOH levels

Mice were randomly divided into 5 groups with 3–6 mice in each to receive 0.4 ml of 15%, 20%, 25%, 30% and 0.5 ml of 30% EtOH in water by gavage as previously described in our studies¹⁴. Mice were sacrificed at various time points after EtOH administration. Blood was drawn via cardiac puncture into heparinized tubes and plasma was collected by centrifuging at 8,000 rpm at 4°C for 10 min. EtOH levels in the plasma were determined¹⁴. The results as summarized in Fig. 1 indicate that the blood EtOH levels were dose dependently increased within one hour in mice after its ingestion. The blood EtOH levels were in the range of 122 ± 19.7 mg/dL in mice gavaged with 0.4 ml of 15% EtOH and 231 ± 24 mg/dL in the group receiving 0.5 ml of 30% EtOH at this time point. The blood EtOH levels returned to basal value in mice given 0.4 ml of 15–20% EtOH within 4 hours after the gavage, however, blood EtOH levels remained above 100 mg/dL in mice gavaged with higher EtOH doses. Regardless of the EtOH dose, nearly 90–95% of the circulating EtOH was metabolized within 8 hours of its administration. We elected to use 0.4 ml of 25% EtOH (2.9g/Kg) in all subsequent experiments, since mice gavaged with this dose showed blood

EtOH levels in the range of 90–100 mg/dL at 4 hours after EtOH administration. This blood EtOH level is within the range of legal limits and comparable to clinical findings as well as similar to our previous studies performed in the rat model of EtOH intoxication and burn injury^{8, 14, 15}.

Mouse model of acute alcohol intoxication and burn injury

Briefly, mice were randomly divided into four groups: sham vehicle; sham EtOH; burn vehicle and burn EtOH. In the EtOH-treated group, mice were gavaged with 0.4 ml of 25% EtOH in water. In the vehicle group, mice were gavaged with 0.4 ml of water. At 4 hours after the gavage, mice were anesthetized with a mixture of ketamine and xylazine at a dose of 79.7 mg/Kg and 1.18 mg/Kg, respectively, by intraperitoneal injection and the dorsal surface was shaved. The mice were transferred into a template, which was fabricated to expose 12–15% of the total body surface area (TBSA). TBSA was calculated by using Walker and Mason formula^{16, 17}. For burn injury, the template with mice was immersed into hot water maintained at 85–90°C for 7–8 sec¹⁷. For sham injury, mice received identical anesthesia and were shaved but were immersed in lukewarm water for 7–8 sec. All animals were dried immediately and resuscitated with 1.0 ml physiological saline by intraperitoneal injection.

Tissue harvesting

Mice were sacrificed on day one after injury. Blood was drawn via cardiac puncture into heparinized tubes and centrifuged (8,000 rpm, 10 min, 4°C). Plasma was collected and stored at –80°C for further analysis. Lung, distal part of small intestine and the liver were removed aseptically and were either stored in liquid nitrogen or fixed in 10% formalin for further analysis. In addition, a portion of the tissues from each organ was used to determine edema formation as described below.

Preparation of tissue homogenates

For the measurement of inflammatory mediators, equal weights of tissues (100 mg) from various groups were sonicated in 1 ml of phosphate buffer saline containing protease inhibitor cocktail (Sigma Chemical Co., St Louis, MO)^{18–20}. For the measurement of myeloperoxidase (MPO) activity, equal weights of tissues (100 mg) were sonicated in 1 ml buffer containing (0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer, pH 6.0)^{18–20}. Homogenates were cleared by centrifuging at 10,000 rpm at 4°C for 15 min. The supernatants were stored at –80°C for the analysis of MPO. Tissue MPO activity and cytokine levels were normalized to the total protein in the homogenates, which was determined by using the BioRad assay kit (Hercules, CA).

Measurement of inflammatory cytokines in blood and tissue homogenates

IL-6, IL-10, MCP-1 and IL-18 levels in blood and tissue homogenates were determined by using ELISA kits according to manufacture's instructions.

Measurement of MPO activity

MPO activity in the tissue homogenates was measured as described previously^{18, 19}. Briefly, the reaction was carried out in a 96-well plate by adding 290 µl 50 mM potassium phosphate buffer, 3 µl substrate solution (containing 20 mg/ml *o*-dianisidine hydrochloride), and 3 µl H₂O₂ (20 mM). Samples (10 µl) were added to the wells to start the reaction. Standard MPO (Sigma Chemical Co.) was used in parallel to determine MPO activity in the sample. The reaction was stopped by adding 3 µl sodium azide (30%). Plates were read at 460 nm. MPO activity was determined by using the curve obtained from the standard MPO and normalized to the protein contents.

Measurement of tissue edema

Tissue edema was measured by determining the tissue water content. In brief, lung, small intestine and liver from various experimental groups were removed, weighed, and dried for 48 hours at 80°C^{18, 19}. Water content (%) of tissue was calculated as (wet wt–dry wt)/wet wt × 100%.

Histological analysis of neutrophil infiltration in organs

Lung, small intestine and liver tissues were fixed in 10% formalin for 24 hours and were sent to Comparative Pathology Laboratory at the University of Alabama at Birmingham for further processing. Briefly, the tissues were embedded in paraffin. These were then cut (4–5 µm thick) and mounted on glass slides. Tissue sections were stained with hematoxylin-eosin (H&E) and observed under the microscope (Nikon Eclipse TS100) at a magnification of 1000× for morphology and neutrophil accumulation in different organs and photographed using a camera (SPOT, RTcolor, Diagnostic Instrument, Inc, Iowa City, Iowa) attached with the microscope^{20, 21}.

Statistical analysis

Results are presented as means ± SEM and were analyzed using ANOVA. The significance between the groups was determined using Tukey's test (GraphPad InStat). A $p < 0.05$ between two groups was considered statistically significant.

Result

EtOH intoxication prior to burn injury enhances IL-6, IL-10 and IL-18 levels

IL-6 was undetectable in circulation of sham animals regardless of EtOH exposure (Fig. 2). Furthermore, no differences in IL-6 levels was found in the small intestine, lung and liver tissue in sham animals following EtOH intoxication compared to shams vehicle group. The levels of IL-6 were significantly elevated in circulation and in other organs following burn injury alone compared to shams. IL-6 levels were several folds higher in circulation in mice receiving ethanol and burn injury compared to shams. An approximately 2-fold increase in IL-6 levels was observed in the intestine, lung and liver in animals receiving a combined insult of EtOH intoxication and burn injury compared to sham injury regardless of EtOH intoxication. Moreover, IL-6 levels were significantly higher in all 4 compartments in mice receiving a combined insult of EtOH intoxication and burn injury compared to those, receiving burn injury alone.

In contrast to IL-6, IL-10 (Fig. 3) and IL-18 (Fig. 4) were not detected in circulation in any of the experimental groups. Additionally, no difference in the IL-10 and IL-18 levels was noted in the small intestine, lung and liver tissues on day one after EtOH intoxication or burn injury alone compared to shams gavaged with vehicle. However, ~2-fold increase in IL-10 levels in the small intestine was observed in mice receiving a combined insult of EtOH intoxication and burn injury compared to mice receiving either insult alone (Fig. 3). Similarly, although a less than 2-fold but a significant increase in IL-10 levels was observed in the lung and liver tissues following a combined insult of EtOH intoxication and burn injury compared to mice receiving either insult alone. The levels of IL-18 on the other hand were found to be nearly 3-fold high in the small intestine and lung, and 2-fold in the liver following EtOH intoxication and burn injury compared to shams (Fig. 4). As compared to burn vehicle group, IL-18 levels remain significantly higher in mice receiving combined insult of EtOH intoxication and burn injury.

EtOH intoxication prior to burn injury enhances MCP-1 levels

As compared to sham vehicle, there was no change in MCP-1 levels in the circulation, small intestine, lung and liver tissues following EtOH exposure or burn injury alone. However, a significant increase in MCP-1 levels in all four tissue compartments was observed in mice receiving a combined insult of EtOH intoxication and burn injury compared to mice receiving either insult alone (Fig. 5).

EtOH intoxication prior to burn injury enhances MPO activity and neutrophil infiltration

As shown in Fig. 6, no difference in MPO activity was observed in the small intestine, lung and liver following EtOH exposure or burn injury alone compared to sham vehicle. A significant increase in MPO activity in the intestine, lung and liver homogenates was observed following a combined insult of EtOH intoxication and burn injury compared to either insult alone. In addition to MPO activity, the presence of neutrophil infiltration in the intestine, lung and liver tissue was further confirmed by H&E staining. H&E stained sections were examined at $\times 1000$ magnification and neutrophils were identified by their distinct morphological feature such as small, pale granular cytoplasm with a multi-lobed nucleus. Photomicrographs shown in Fig. 7 clearly demonstrate the presence of neutrophils in most of the groups in the small intestine (Fig. 7A), lung (Fig. 7B) and liver (Fig. 7C). However, the number of neutrophil was much higher in mice receiving a combined insult of EtOH intoxication and burn injury compared to either insult alone (Table 1).

EtOH intoxication prior to burn injury enhances tissue edema

As compared to sham vehicle group, no change in water content in all the three organs was observed following either EtOH intoxication or burn injury alone. However, EtOH intoxication combined with burn injury resulted in a significant increase in edema in the small intestine, lung and liver compared to either shams or burn injury alone (Fig. 8).

Discussion

We found that EtOH intoxication combined with burn injury increases tissue edema in the intestine, liver and the lung compared to either EtOH intoxication or burn injury alone. This was accompanied with an increase in cytokines, chemokines and neutrophil infiltration. Several lines of evidence indicate that trauma, burn and sepsis initiate a cascade of events which is characterized by uncontrolled production of inflammatory mediators often referred to as systemic inflammatory response syndrome (SIRS) ^{22–24}. This release of cytokines or inflammatory mediators is known to precede organ dysfunction and failure. Although numerous advances in intensive care units and patient management have improved short-term survival, sepsis and subsequent multiple organ failure continue to be the major cause of morbidity and mortality in patients who survive the initial injury.

Previous studies have suggested that IL-6 is a multifunctional cytokine ^{25–27}. In the early phase of infection, IL-6 induces B cell proliferation, T cell activity and neutrophil infiltration and thus becomes an important player in mediating the immune responses initiated by infection and injury ^{25–27}. However, high level of IL-6 has been widely used as a marker of the severity of inflammatory response in many clinical conditions ^{28–30}. We observed that IL-6 was undetectable in circulation in sham animals. However, a significant increase in IL-6 was observed in circulation, lung, small intestine and liver tissue following burn injury with or without prior EtOH intoxication compared with shams; but the levels were higher in the group of mice receiving a combined insult of EtOH intoxication and burn injury. Consistent with this observation, other studies also demonstrate that the combination of acute ethanol and burn injury lead to a substantial increase in IL-6 levels in the serum and other organs ^{25, 31, 32}. Similarly, IL-18 also possesses broad immunomodulatory properties,

including a role in tissue damage. IL-18 is often expressed in inflammatory lesions and is found to be elevated in the blood of patients with a variety of diseases including Crohn's disease, Sjogren syndrome, graft-versus-host disease and rheumatoid arthritis³³. Furthermore, a significant increase in IL-18 levels in circulation was also observed in patients with lethal sepsis compared with sepsis survivors at all time-points studied³⁴. These studies suggested that inappropriate IL-18 release contributes to the pathogenesis of these diseases and may influence the clinical outcome in these patients^{34–36}.

An increase in IL-6 and IL-18 in the small intestine has been correlated with decreased villus height in the small intestine following EtOH intoxication and burn injury^{20, 31}. Furthermore, both IL-6 and IL-18 are also implicated in lung edema following EtOH intoxication and burn injury^{21, 37}. However, the mechanism by which increased IL-6 and IL-18 in mice given EtOH and burn injury causes tissue damage in 3 different organs remains largely unknown. One potential common denominator to these cytokines is the recruitment of neutrophils to different organs observed in these mice. Earlier studies in the past have shown that the depletion of neutrophil prior to injury protects organ function following burn and trauma-hemorrhage^{38, 39}. Consistent with these findings we also observed that depletion of neutrophil prevent the increase in intestine tissue damage following EtOH and burn injury⁴⁰. We showed recently that IL-18 plays a critical role in neutrophil superoxide anion production and their recruitment to lung and intestine following EtOH and burn injury^{20, 21, 40}. A similar role for IL-18 was demonstrated in experimental models of sepsis⁴¹. In another study, intratracheal administration of IL-18 resulted in increased neutrophil infiltration and lung vascular permeability³⁵. Thus, it is likely that these cytokines directly or via up-regulation of chemokines such MCP-1 may cause an increase in the neutrophil recruitment. MCP-1 is known to recruit monocytes⁴²; however, there is evidence that MCP-1 enhances intercellular adhesion molecule-1 (ICAM-1) expression⁴³, a step critical to the recruitment of neutrophils. Thus MCP-1 may facilitate the neutrophil recruitment via ICAM-1. Similarly IL-6 and IL-18 is shown to up-regulate the expression of ICAM-1 and other chemokines critical to neutrophil recruitment.

Regardless the mechanisms, once the neutrophils are recruited, they may contribute to the tissue damage (i.e., edema formation) observed in mice given EtOH and burn injury. Neutrophils are among the first line of defense against invading pathogens and thus they recruit to the site of inflammation within a short period of time after injury^{21, 38, 40, 44}. After destroying the invading pathogens, neutrophils undergo apoptosis and are cleared by macrophages. In a recent study, we observed that EtOH combined with burn injury prolonged their presence in intestine^{40, 44}. Whether this increase in neutrophil presence is due to a delay in their apoptosis or an increase in their flux remains to be established. Nevertheless, their prolong presence in the tissue may result in increased O_2^- and proteases accumulation. Although such an increase in O_2^- and proteases should be helpful in killing bacteria and other invading pathogens, their prolonged presence and accumulation in excess are considered to be the major determinants in tissue damage in multiple organs in various inflammatory conditions^{45–47}.

In this study, we used a small area burn injury (12–15%) which by itself only increases IL-6 levels in blood, small intestine, lung and liver tissue, but did not influence other parameters on day one after injury. However, EtOH intoxication combined with burn resulted in significant increase in cytokines and chemokines, neutrophil infiltration and tissue edema. A previous study has shown that burn injury size is a critical factor in postburn pathogenesis⁹ and while a small burn itself may not cause a significant change, other existing factors, such as age, gender and preclinical conditions may influence the outcome following small burn injury. Thus, whereas a smaller burn injury by itself may not have an adverse effect on host defense, when combined with EtOH intoxication it may become detrimental. We also

recognize that in contrast to a previous study³¹, we found an increase in intestinal IL-10 levels following a combined insult of EtOH intoxication and burn injury. The definitive cause for these differences remains to be established. However, these differences could result from the different models employed in the two studies. In the present study, EtOH was administered by gavage and the burn injury was induced at 4 hours after its administration. The previous study on the other hand utilized a model in which EtOH was administered intraperitoneally and burn injury was induced 30 minutes after EtOH administration.

In summary, our findings suggest that acute EtOH intoxication prior to burn injury lead to an increase in inflammatory cytokines and chemokines such as IL-6, IL-10, MCP-1 and IL-18. This was accompanied with an increase in neutrophil infiltration and edema formation in more than one organ. These findings form the basis for future studies investigating the mechanism by which EtOH combined with burn injury causes tissue damage.

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Fig. 1. Circulatory EtOH levels in mice

Mice were gavaged with EtOH and sacrificed at various time points thereafter. Blood was drawn via cardiac puncture and plasma EtOH levels were measured by using EtOH detection kit. Values are mean $\pm$ SEM from 3–6 animals in each group

Fig. 2. IL-6 levels in circulation, small intestine, lung and liver after EtOH and burn injury

On day one after injury, blood, intestine, lung and liver were collected from various experimental groups. Tissues were homogenized and IL-6 levels in the tissue homogenates and plasma were determined by using ELISA kit. Values are mean $\pm$ SEM from 4–7 animals per group. *, $p < 0.05$ compared with others. #, $p < 0.05$ compared with sham vehicle.

Fig. 3. IL-10 levels in small intestine, lung and liver after EtOH and burn injury

One day after injury, intestine, lung and liver were collected and homogenized. IL-10 levels in the tissue homogenates were determined by using ELISA kit. Values are mean $\pm$ SEM from 4–7 animals in each group. *, $p < 0.05$ compared with other groups.

Fig. 4. IL-18 levels in small intestine, lung and liver after EtOH and burn injury

One day after injury, intestine, lung and liver were collected and homogenized. IL-18 levels in the tissue homogenates were determined by using ELISA kit. Values are mean $\pm$ SEM from 4–7 animals in each group. *, $p < 0.05$ compared with other groups.

Fig. 5. MCP-1 levels in circulation, small intestine, lung and liver after EtOH and burn injury
One day after injury, blood, intestine, lung and liver were collected. MCP-1 levels in the tissue homogenates and plasma were determined by using ELISA kit. Values are mean $\pm$ SEM from 4–7 animals in each group. *, $p < 0.05$ compared with other groups.

Fig. 6. MPO activity in small intestine, lung and liver after EtOH and burn injury

One day after injury, intestine, lung and liver were collected and homogenized. MPO levels in the tissue homogenates were determined and normalized to the protein. Values are mean $\pm$ SEM from 4–7 animals in each group. *, $p < 0.05$ compared with other groups.

Fig. 7. Histological analysis of neutrophil infiltration in small intestine, lung and liver after EtOH and burn injury

One day after injury, intestine, lung and liver were collected and processed for hematoxylin-eosin staining. Sections were examined at a magnification of $\times 1000$ and photographed. Representative photomicrographs of intestine (A), lung (B) and liver (C) from four groups shows for the presence of neutrophils. Neutrophils identified by their specific multilobed nucleus are shown by the arrow. Each slide was examined for 3 microscopic fields and each group contained 3 animals.

Fig. 8. Tissue edema in small intestine, lung and liver after EtOH and burn injury

One day one after injury, edema formation in the intestine, lung and liver was determined by assessing the tissue water content. Values are mean $\pm$ SEM from 4–7 animals in each group.

*, $p < 0.05$ compared with other groups.

Table 1

The number of neutrophil in small intestine, lung and liver after EtOH intoxication and burn injury[§]

	Sham vehicle	Sham EtOH	Burn vehicle	Burn EtOH
Intestine	1.56 ± 0.24	1.78 ± 0.28	2.67 ± 0.37	4.11 ± 0.46*
Lung	8.11 ± 0.89	9.11 ± 0.86	11.78 ± 0.81 [#]	19.11 ± 1.09*
Liver	2.11 ± 0.45	2.22 ± 0.36	3.11 ± 0.39	8.67 ± 1.00*

[§] Small intestine, lung and liver tissue sections were stained with hematoxylin-eosin (H&E) and examined under the microscope at 1000× magnification. Neutrophils were identified by their distinct morphological feature such as small, pale granular cytoplasm with a multi-lobed nucleus. Each section was scanned for 3 microscopic fields, and the data thus obtained from 3 animals in each group were pooled and presented as mean ± SEM per single high powered microscopic field.

* p < 0.05 compared with other groups.

[#] p < 0.05 compared with sham vehicle.