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Ethanol-Induced Macrophage Apoptosis: The Role of TGF-β

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Both clinical and laboratory reports indicate that ethanol addicts are prone to recurrent infections. We hypothesize that ethanol promotes macrophage apoptosis, thus compromising the efficiency of the mononuclear phagocyte system in dealing with infection. We studied the effect of ethanol on macrophage apoptosis. Human monocytes isolated from healthy subjects after an alcohol drinking binge showed enhanced apoptosis (before, 1.2 ± 0.3% vs after, 28.4 ± 3.7% apoptotic cells/field). Peritoneal macrophages harvested from ethanol-treated rats also showed increased (p < 0.0001) apoptosis. DNA isolated from peritoneal macrophages of ethanol-treated rats displayed integer multiples of 200 base pairs (ladder pattern). Furthermore, macrophages harvested from ethanol-treated rats had an enhanced expression as well as accumulation of TGF-β. In in vitro studies, ethanol promoted apoptosis of human monocytes as well as rat peritoneal macrophages. In addition, ethanol enhanced apoptosis of murine macrophages (J774) in a time-dependent manner. The ethanol-induced apoptosis was amplified by LPS and partly attenuated (p < 0.001) by anti-TGF-β Ab. TGF-β also promoted macrophage apoptosis in a dose-dependent manner. Moreover, ethanol enhanced TGF-β protein production by macrophages. These results indicate that ethanol promotes macrophage apoptosis. This effect of ethanol seems to be partly mediated through the generation of TGF-β by macrophages. The Journal of Immunology, 1999, 162: 3031–3036.

Medical history on the use of ethanol indicates that alcoholics are prone to infections (1). As early as in 1785, Benjamin Rush suggested that the use of ethanol was associated with tuberculosis, pneumonia, and yellow fever (2). In 1884, Koch described that most patients developing cholera were excessive drinkers and demonstrated that ethanol-administered rats were more susceptible to develop cholera (3). Since then, both clinical and laboratory reports have demonstrated that ethanol modulates the immune function directly as well as indirectly through malnutrition and cirrhosis (4–7). Ethanol modulates the function of polymorphonuclear cells, lymphocytes, and macrophages (8–11).

Various investigators have demonstrated a decreased clearance rate of bacteria from the lungs of ethanol-treated animals (12). This effect of ethanol has been predominantly attributed to altered macrophage function including decreased mobilization into the lung and a decrease in their adherence, phagocytosis, and bactericidal activity (13–16). In vivo studies have also shown a decreased rate of bacterial clearance by peritoneal macrophages (17). In vitro study documentation shows that ethanol compromises the phagocytic capability of macrophages (18, 19).

We hypothesize that ethanol programs macrophages to die (apoptosis). In an ethanol milieu, a significant percentage of macrophages may be apoptosed, limiting their participation in migration, adherence, and phagocytosis. In the present study, we examined the effect of ethanol on macrophage apoptosis.

It has been demonstrated that ethanol can stimulate macrophages to generate TGF-β (20, 21). Because TGF-β has been reported to promote apoptosis in a variety of cells (22, 23), we asked whether ethanol-induced macrophage apoptosis is mediated through the generation of TGF-β.

Materials and Methods

Experimental animals

Twelve Sprague-Dawley rats (Crl:CD[SD]BR-C; Charles River Laboratories, Wilmington, MA) were administered i.p. either PBS (control, six rats) or PBS containing ethanol (20%, 5.5 g/kg body weight, six rats weighing 180–200 g) every 8 h for 24 h. To determine whether acute ethanol administration in rats also results in elevated blood ethanol levels, four additional rats were administered i.p. PBS containing ethanol (5.5 g/kg body weight). After 1 h of ethanol administration, blood was collected and ethanol concentration in the blood was measured.

Rat peritoneal macrophage preparations

Rat peritoneal macrophages were isolated from the above designated rats as described previously (24). More than 95% of the harvested macrophages stained positively with ED-1.

Murine macrophage culture

To determine the species-specific effect of ethanol on macrophages, we studied the effect of ethanol on murine macrophages (J774, American Type Culture Collection, Manassas, VA). Confluent macrophages were subcultured in DMEM (Life Technologies, Grand Island, NY) containing 10% FCS (Life Technologies), 50 µg/ml of penicillin, and 50 µg/ml of streptomycin (Life Technologies).

Human monocytes

Human monocytes were isolated from healthy volunteers as described previously (24). To evaluate the effect of ethanol on monocyte apoptosis in vivo, 10 ml of blood from healthy volunteers before and after an alcohol drinking binge was collected and monocytes were harvested.

Apoptosis studies

In in vitro studies, we evaluated this effect of ethanol in concentrations that were usually observed after an excessive intake of alcohol in normal individuals (50–100 mM) and after acute ethanol intake in chronic alcoholics.
(150 mM) (25). To determine the occurrence of apoptosis and necrosis in macrophages, we used Hoechst (H-33342, Molecular Probes, Eugene, OR) and propidium iodide (Sigma, St. Louis, MO). H-33342 stains the nuclei of live cells and identifies apoptotic cells by increased fluorescence. On the contrary, propidium iodide stains the necrosed cells. Subconfluent macrophages were prepared under various conditions. At the end of the incubation period, aliquots of methanol containing H-33342 (final concentration, 1 µg/ml) were added and the cells were incubated for 10 min at 37°C. Subsequently, cells (without a wash) were placed on ice, and propidium iodide (final concentration, 1 µg/ml) was added to each well. Cells were incubated with the dyes for 10 min on ice, protected from light, and then examined under UV light using a Hoechst filter (Nikon, Melville, NY). The percentage of live, apoptotic, and necrosed cells were recorded in eight random fields by two observers unaware of the experimental conditions.

**DNA isolation and gel electrophoresis**

Equal numbers of peritoneal macrophages were isolated from control and ethanol-treated rats, lysed in DNA lysis buffer, and DNA was extracted (24) and run on a 1.8% agarose gel and electrophoresed at 5 V/cm in 0.5× TE buffer (Tris 10 mM; EDTA 1 mM, pH 8.0) containing 10 µg/ml ethidium bromide.

**RNA extraction and Northern blotting**

To determine the effect of ethanol on mRNA expression of TGF-β, equal numbers of peritoneal macrophages harvested from control and ethanol-treated rats were lysed, and total RNA was extracted (24). Northern blots were generated and probed with cDNA specific for TGF-β (American Type Culture Collection) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Densitometric analysis was performed and the ratio of TGF-β:GAPDH was determined.

**Immunocytochemical labeling**

Peritoneal macrophages isolated from control and ethanol-treated rats were plated on chamber slides and unattached cells were washed out with 20 mM phosphate buffer, pH 7.5. Attached cells were fixed in 100% methanol at −20°C for 5 min and then incubated with normal goat serum (1:20; Vector Laboratories, Burlingame, CA) followed by incubation with rabbit polyclonal Ab to TGF-β1 (1:150, Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min and washed with phosphate buffer three times. The cells were incubated with a secondary Ab, which was a biotinylated goat anti-rabbit IgG (1:150; Vector Laboratories), for 60 min at 25°C. It was followed by the addition of a 0.1 M solution of 3,3-diaminobenzidine (Sigma) in 0.05 M Tris (hydroxymethyl)aminomethane buffer, pH 7.6 (5 min). Slides were counterstained with 1% methyl green solution for 20 min and mounted with glass coverslips and a drop of permount. Slides were examined under a light microscope by two observers unaware of the experimental conditions.

**Statistical analysis**

For comparison of mean values between two groups, an unpaired t test was used. To compare values between multiple groups, ANOVA was applied. A Newman-Keuls multiple range test was used to calculate a q value. All values are reported as mean ± SEM.

**Results**

**Effects of ethanol on murine and rat peritoneal macrophage apoptosis**

Acutely ethanol-intoxicated rats showed a mean blood ethanol concentration of 125 mM. Rats were lethargic after the administration of ethanol. However, the administration of one-third of this dose made the rats a little inactive, but they remained functional. Peritoneal macrophages isolated from ethanol-treated rats showed greater (p < 0.0001) apoptosis when compared with macrophages isolated from control rats (control, 1.6 ± 0.2%; ethanol, 12.9 ± 0.2% apoptotic macrophages/field).

DNA isolated from macrophages harvested from ethanol-treated rats displayed a classic ladder pattern as shown in Fig. 1.

To determine the dose-response effect of ethanol on peritoneal macrophage apoptosis, peritoneal macrophages were isolated from control rats. Macrophages were plated in 24-well plates in medium (DMEM plus 5% FCS) containing either vehicle (control) or various concentrations of ethanol (50, 100, 150, and 200 mM) for 24 h. Four to 12 series of experiments were conducted. The dose response effect of ethanol on peritoneal macrophage apoptosis is shown in Fig. 2. Ethanol at concentrations of 100 mM and higher promoted macrophage apoptosis (control, 5.5 ± 1.0; 100 mM ethanol, 14.3 ± 1.1%; 150 mM ethanol, 29.9 ± 1.8%; 200 mM ethanol, 39.4 ± 3.8% apoptotic macrophages/field). Ethanol at a concentration of 150 mM also promoted macrophage necrosis (control, 1.7 ± 0.6% vs 150 mM ethanol, 20.7 ± 1.5%; 200 mM ethanol, 36.2% necrosed macrophages/field).

To determine the species-specific difference in the effect of ethanol on macrophage apoptosis, we used a murine macrophage cell line (J774). Equal numbers (10,000 cells/well) of macrophages (J774) were plated on 24-well plates and grown to subconfluence.

**FIGURE 1.** Gel electrophoresis of DNA isolated from peritoneal macrophages of control rats (lanes 2, 4, and 6) and ethanol-treated rats (lanes 3, 5, and 7). Molecular markers are as shown (lanes 1 and 8). Macrophages harvested from ethanol-treated rats (lanes 3, 5, and 7) showed a classic ladder pattern.

**FIGURE 2.** Dose-response effect of ethanol on peritoneal macrophage apoptosis. Peritoneal macrophages were plated in 24-well plates in medium (DMEM plus 5% FCS) containing either vehicle (control) or variable concentrations of ethanol (50, 100, 150, and 200 mM) for 24 h. At the end of the incubation period, cells were stained with H-33342 and propidium iodide, and the percentage of live, apoptotic, and necrosed cells was counted. Results (mean ± SEM) are from 4–12 series of experiments. To compare values between multiple groups, ANOVA was applied. A Newman-Keuls multiple range test was used to calculate a q value. *, p < 0.001 compared with control and 50–100 mM ethanol; **, p < 0.001 compared with control and 50–100 mM ethanol; ***, p < 0.001 compared with control and 50 mM ethanol; ****, p < 0.01 compared with 150 mM ethanol.
Cells were washed twice with PBS and incubated in medium (DMEM plus 1% FCS) containing either vehicle (control) or variable concentrations of ethanol (50, 100, 150, and 200 mM) for 24 h. At the end of the incubation period, cells were stained with H-33342 and propidium iodide, and the percentage of live, apoptotic, and necrosed cells was recorded. Results (mean ± SEM) are from eight sets of experiments, each conducted in triplicate. To compare values between multiple groups, ANOVA was applied. A Newman-Keuls multiple range test was used to calculate a q value. *, p < 0.001 compared with control and 50–150 mM ethanol; **, p < 0.001 compared with control and 50–100 mM ethanol; ***, p < 0.001 compared with control; ****, p < 0.01 compared with control.

To evaluate whether human monocytes are also susceptible to the effect of ethanol, freshly isolated human monocytes were incubated with variable concentrations (0, 50, 100, 150, and 200 mM) of ethanol for 24 h. Four to eight series of experiments were conducted. The effect of ethanol on human monocyte apoptosis is shown in Fig. 5. Ethanol in concentrations of 100 mM or higher enhanced apoptosis of human monocytes (control, 1.1 ± 0.2%; 100 mM ethanol, 9.8 ± 0.5%; 150 mM ethanol, 16.6 ± 1.0%; 200 mM ethanol, 28.5 ± 0.7% apoptotic monocytes per/field).

In in vivo studies, binge drinking of alcohol by healthy volunteers enhanced apoptosis of monocytes (before, 1.2 ± 0.3% vs after binge drinking, 28.4 ± 3.7% apoptotic monocytes/field; p < 0.001).

To determine the time course of ethanol effect on macrophage apoptosis, equal numbers of subconfluent macrophages (J774) were incubated in medium (DMEM plus 1% FCS) containing either vehicle (control) or ethanol (150 mM) for variable time periods (4, 8, 16, and 24 h). Four series of experiments were conducted, each in triplicate. As shown in Fig. 6, ethanol enhanced macrophage apoptosis in a time-dependent manner.

**Effect of LPS on murine macrophage apoptosis**

Because ethanol has been demonstrated to promote the generation of cytokines by LPS-activated macrophages, we evaluated the effect of LPS on ethanol-induced macrophage apoptosis. Equal numbers of subconfluent macrophages (J774) were incubated in medium (DMEM plus 1% FCS) containing either vehicle (control), LPS (10 ng/ml, control), or variable concentrations of ethanol (75 and 150 mM) ± LPS (10 ng/ml) for 24 h. Four sets of experiments were performed, each set in triplicate. The effect of LPS on ethanol-induced macrophage apoptosis is shown in Fig. 7. LPS amplified the effect of ethanol on macrophage apoptosis (control, 2.1 ± 0.5%; 150 mM ethanol, 14.2 ± 0.3%; 150 mM ethanol plus LPS, 21.5 ± 1.2% apoptotic macrophages/field).

**Effects of ethanol on murine and peritoneal macrophage TGF-β expression**

To determine the effect of ethanol on the cytoplasmic content of TGF-β, equal numbers of subconfluent macrophages (J774) grown...
cytoplasmic staining for TGF-β. Isolated macrophages harvested from ethanol-treated animals showed a greater increase in mRNA expression of TGF-β.

**Effects of TGF-β on macrophage apoptosis**

To determine the effect of TGF-β on macrophage apoptosis, equal numbers of subconfluent macrophages (J774) were incubated in medium (DMEM plus 1% FCS) containing either vehicle (control) or variable concentrations of TGF-β (0.1, 1, 5, 10, and 25 ng/ml) for 24 h. Four series of experiments were conducted, each in triplicate. Results are shown in Fig. 9. TGF-β promoted macrophage apoptosis in a dose-dependent manner (control, 1.0 ± 0.3%; 0.1 ng/ml TGF-β, 3.3 ± 0.5%; 1 ng/ml TGF-β, 4.8 ± 0.1%; 5 ng/ml TGF-β, 10.5 ± 0.7%; 10 ng/ml TGF-β, 14.3 ± 0.5%; 25 ng/ml TGF-β, 28.1 ± 0.4% apoptotic macrophages/field).

**Role of TGF-β on ethanol-induced macrophage apoptosis**

Because ethanol has been demonstrated to stimulate the expression of TGF-β by macrophages, we evaluated the role of TGF-β in ethanol-induced macrophage apoptosis. Equal numbers of subconfluent macrophages (J774) were incubated in medium (DMEM plus 1% FCS) containing either vehicle (control), anti-TGF-β Ab (5 μg/ml; rabbit; Santa Cruz Biotechnology), rabbit IgG (5 μg/ml; Vector Laboratories), nonimmune rabbit serum (5 μg/ml; Vector Laboratories), ethanol (150 mM), ethanol (150 mM) plus anti-TGF-β Ab (5 μg/ml), ethanol (150 mM) plus IgG (5 μg/ml), or ethanol (150 mM) plus serum (5 μg/ml) for 24 h. Four sets of experiments were conducted, each in triplicate. The effect of anti-TGF-β Ab on ethanol-induced apoptosis is shown in Fig. 10.
Ethanol enhanced \((p < 0.001)\) macrophage apoptosis. Anti-TGF-\(\beta\) Ab partly attenuated \((p < 0.001; 56\%)\) the ethanol-induced macrophage apoptosis (control, 0.7 \(\pm\) 0.6%; 150 mM ethanol, 23.5 \(\pm\) 0.8%; anti-TGF-\(\beta\) Ab, 0.6 \(\pm\) 0.6%; IgG, 1.1 \(\pm\) 0.6%; serum, 1.8 \(\pm\) 0.6%; 150 mM ethanol plus anti-TGF-\(\beta\) Ab, 13.3 \(\pm\) 1.4%; 150 mM ethanol plus IgG, 21.5 \(\pm\) 1.2%; 150 mM ethanol plus serum, 21.3 \(\pm\) 1.0% apoptotic macrophages/field).

Gel electrophoresis on DNA isolated from macrophages (J774) incubated in medium containing ethanol \(\pm\) LPS with or without anti-TGF-\(\beta\) Ab for 24 h further confirms our findings (data not shown). Ethanol (150 mM)-treated macrophages showed a classic ladder pattern. Control macrophages and macrophages treated with anti-TGF-\(\beta\) Ab showed no DNA fragmentation. LPS-treated macrophages showed minimal DNA fragmentation. However, LPS amplified the effect of ethanol on macrophage DNA fragmentation. Anti-TGF-\(\beta\) Ab attenuated ethanol (150 mM)-induced macrophage apoptosis. Cells treated with ethanol showed a classic ladder pattern in the form of integer multiples of 200 base pairs. However, anti-TGF-\(\beta\) Ab attenuated this effect of ethanol on macrophages. These results suggest that ethanol-induced apoptosis may be mediated through the generation of TGF-\(\beta\) by macrophages.

**Discussion**

The present study demonstrates that ethanol promotes apoptosis of macrophages. This effect of ethanol was time dependent. Ethanol also promoted apoptosis of human monocytes. LPS amplified the effect of ethanol on macrophage apoptosis. Ethanol enhanced macrophage mRNA expression of TGF-\(\beta\) as well as cytoplasmic TGF-\(\beta\) content. Because anti-TGF-\(\beta\) Abs attenuated ethanol-induced macrophage apoptosis, it appears that ethanol-induced macrophage apoptosis may be partly mediated through the generation of TGF-\(\beta\).

The mononuclear cell phagocyte system is an important defense mechanism against a variety of pathogenic microorganisms (26). Phagocytosis of bacteria is initiated with the binding to surface receptors (Fcr or C3b), clustering of receptors in localized domains, and their internalization in a vesicle followed by transport to endosomes and lysosomes (27). In these organelles, the interaction of microorganisms with enzymes leads to the release of a variety of reactive oxygen species that may be microbicidal (28). Bagasra et al. demonstrated that the ability of macrophages from alcoholic rats to phagocytose through C3b and Fc receptors was attenuated (29). Similarly, the ability of peritoneal macrophages to phagocytose nonopsonized Candida albicans was impaired (29).

In in vitro studies, ethanol incubation attenuated the phagocytic capability of human monocytes for latex particles and SRBC coated with IgG (19, 30). Isolated perfused liver from rats fed ethanol for 3 wk showed a decreased clearance and killing of perfused bacteria (31). Intact rats also showed a decreased clearance of microaggregated albumin (32). Similarly, the clearance of i.v. administered aggregated serum albumin was slower in 12 alcohols (lacking evidence of cirrhosis) within 24 h of detoxification when compared with normal subjects (33). In the present study, ethanol induced macrophage apoptosis in vitro as well as in vivo. We may suggest that in an ethanol milieu a certain percentage of

**FIGURE 9.** Effect of TGF-\(\beta\) on murine macrophage apoptosis. Equal numbers of subconfluent macrophages (J774) were incubated in media (DMEM plus 1% FCS) containing variable concentrations of TGF-\(\beta\) (0, 0.1, 1, 5, 10, and 25 ng/ml) for 24 h. At the end of the incubation period, cells were stained with H-33342 and propidium iodide. The percentage of live and apoptotic cells was counted. Results (mean \(\pm\) SEM) are from four series of experiments, each conducted in triplicate. \(*, p < 0.001\) compared with 0–1 ng/ml TGF-\(\beta\); \(p p p, p < 0.001\) compared with 0–1 ng/ml TGF-\(\beta\); \(a, p < 0.05\) compared with control; \(b, p < 0.001\) compared with control.

**FIGURE 10.** Effect of anti-TGF-\(\beta\) Ab (TGF-ab) on ethanol-induced macrophage apoptosis. Equal numbers of subconfluent macrophages were incubated in medium (DMEM plus 1% FCS) containing either vehicle (control), anti-TGF-\(\beta\) Ab (TGF-ab, 5 \(\mu\)g/ml), ethanol (ETH, 150 mM), isotype IgG (5 \(\mu\)g/ml), nonimmune serum (5 \(\mu\)g/ml), ETH (150 mM) plus TGF-ab (5 \(\mu\)g/ml), ETH (150 mM) plus IgG, or ETH (150 mM) plus serum for 24 h. At the end of the incubation period, cells were stained with H-33342 and propidium iodide. The percentage of live and apoptotic cells was counted. Results (mean \(\pm\) SEM) are from four series of experiments, each conducted in triplicate. To compare values between multiple groups, ANOVA was applied. A Newman-Keuls multiple range test was used to calculate a q value. \(*, p < 0.001\) compared with control, TGF-\(\beta\) ab, IgG, and serum; \(p p p, p < 0.001\) compared with ETH (150 mM), TGF-ab, IgG, and serum; \(p p p, p < 0.001\) compared with ETH plus TGF-ab, IgG, and serum.
macrophages may undergo apoptosis, and these apoptosed macrophages may not be able to migrate to the site of infection. Furthermore, many of the migrated macrophages may also undergo apoptosis and may not be able to participate in the clearance of bacteria by phagocytosing the bacteria. This effect of ethanol on macrophages may compromise the infection-combating power of the host.

TGF-β has been previously demonstrated to induce apoptosis in different cell types (22, 23). The expression of TGF-β mRNA has been demonstrated to increase in castration-induced apoptosis of the rat ventral prostate (34). Estrogen-sensitive breast cancer cells have been demonstrated to increase in castration-induced apoptosis of different cell types (22, 23). The expression of TGF-β has been demonstrated to induce apoptosis in uterine cells, hepatocytes, and osteoclasts (23, 37, 38). Szabo et al. reported the effect of ethanol on macrophage TGF-β production (20, 25). These investigators showed a significant increase of TGF-β production by ethanol-treated macrophages (20, 25). Moreover, pretreatment of macrophages with ethanol augmented TGF-β release in response to activation with the synthetic bacterial analogue, muramyl dipeptide. The amount of TGF-β produced by macrophages in ethanol-stimulated states (20) was within the range of the concentrations that has promoted macrophage apoptosis in the present study.

We conclude that ethanol promotes macrophage apoptosis. This effect of ethanol seems to be mediated through the generation of TGF-β by macrophages. The present study provides a mechanistic insight into the effect of ethanol on the mononuclear phagocyte system.

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