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Alcohol Enhances HIV Infection of Cord Blood Monocyte-Derived Macrophages

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Abstract

Alcohol consumption or alcohol abuse is common among pregnant HIV⁺ women and has been identified as a potential behavioral risk factor for the transmission of HIV. In this study, we examined the impact of alcohol on HIV infection of cord blood monocyte-derived macrophages (CBMDM). We demonstrated that alcohol treatment of CBMDM significantly enhanced HIV infection of CBMDM. Investigation of the mechanisms of alcohol action on HIV demonstrated that alcohol inhibited the expression of several HIV restriction factors, including anti-HIV microRNAs, APOBEC3G and APOBEC3H. Additionally, alcohol also suppressed the expression of IFN regulatory factor 7 (IRF-7) and retinoic acid-inducible gene I (RIG-I), an intracellular sensor of viral infection. The suppression of these IFN regulatory factors was associated with reduced expression of type I IFN. These experimental findings suggest that maternal alcohol consumption may facilitate HIV infection, promoting vertical transmission of HIV.

Keywords

Alcohol; Anti-HIV miRNAs; Cord blood; Intracellular restriction factors; HIV

INTRODUCTION

Primary HIV infection among women of childbearing age has contributed to the global pediatric HIV epidemic [1–3]. In 2011, there were 3.3 million children living with HIV and an estimated 330,000 children who became newly infected with HIV worldwide, primarily through mother-to-child transmission of HIV [2, 4]. In the United States, HIV/AIDS is ranked the third leading cause of death among women aged 25 to 44 years old and accounts for the most common cause of death among African-American women in this age group [5,

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

6]. Since the institution of antiretroviral therapy, the incidence of vertical transmission of HIV in the U.S. and other resource-rich countries has decreased significantly but continues to be a critical issue in resource-poor nations and among poor populations in resource-rich nations. The development of effective preventive strategies against vertical transmission and disease progression in HIV-infected children requires an understanding of the host and environmental factors that influence host innate immunity related to HIV infection. Alcohol consumption and misuse have been identified as potential behavioral risk factors for HIV transmission. Nearly half of pregnant HIV⁺ women drink alcohol [7]. Although it is known that alcohol has immunosuppressive effects [8–12] and may have a cofactor role in promoting HIV infection [13–20], there are still unresolved questions about alterations of the neonatal immune system and its subsequent susceptibility to HIV infection following alcohol exposure.

Neonatal cellular immunity is innately weaker than that of the adult, and therefore, the neonate is at a higher risk for infection. It has been shown that immune cells from neonates are more susceptible to HIV infection than those from adults [6, 21, 22]. It is likely that acute or chronic alcohol exposure can exacerbate neonatal cellular immune system defects. Several *in vitro* studies demonstrated that alcohol has the ability to enhance HIV infection in PBMC [23, 24], T lymphocytes [14, 25] and macrophages [26]. Thus, to determine changes of host cell innate immunity related to HIV infection of neonate macrophages exposed to alcohol is the area of great interest and high significance, which may help to determine previously unidentified mechanisms involved in HIV vertical transmission. In the present study, we evaluated the effect of a clinically appropriate dose of alcohol on neonatal innate immunity and HIV infection of neonatal monocytes/macrophages.

MATERIALS AND METHODS

Neonatal Monocyte Preparations

The study was approved by the Temple University Institutional Review Board. Cord blood was obtained from 6 normal term HIV negative deliveries. Cord blood was layered on a Ficoll gradient and separated by centrifugation at $400 \times g$ for 45 min. The mononuclear layer is collected and incubated with Dulbecco's Modified Eagle's Medium (DMEM) in 2% gelatin-coated flasks for 45 min at 37°C, followed by removal of non-adherent cells with DMEM. The purity (> 97%) of monocytes was determined by fluorescence activated cell sorting analysis using monoclonal antibody against CD14 and low-density lipoprotein specific for monocytes and macrophages. Cells were plated in 48 -well culture plates (2.5×10^5 cells/well) in DMEM containing 10% fetal calf serum (FCS). Monocytes differentiated into macrophages during *in vitro* cultures (5–7 days).

Reagents and HIV Strain

Alcohol was purchased from PHARMCO-AAPER Company (Brookfield, CT). Naltrexone was obtained from Sigma (St Louis, MO). HIV macrophage-tropic strain (Bal) was obtained from the AIDS Research and Reference Reagent Program at the National Institute of Health (NIH, Bethesda, MD).

Alcohol and/or Naltrexone Treatment and HIV Infection

Seven-day-cultured cord blood monocyte derived-macrophages (CBMDM, 2.5×10^5 cells/well) were treated with or without alcohol at different concentrations (10–40 mM) for different time points (3 h, 6 h, and 24 h). The concentrations of alcohol used were based on our previously published *in vitro* dose response studies (10–40 mM) that quantified a maximum biological response without target cell toxicity [25–28]. In order to determine whether the opioid receptor is involved in alcohol-mediated action, the cells were pre-treated with naltrexone (10^{-8} M), a pan-opioid receptor antagonist, for 1 h, followed by alcohol treatment. CBMDM were infected with equal amounts of cell-free HIV Bal (p24 20 ng/ 10^6 cells) for 2 h at 37°C after 24h treatment with or without alcohol. The cells were then washed three times with plain DMEM to remove any unabsorbed virus, and fresh media containing alcohol, was added to the cell cultures. The final wash was tested for HIV reverse transcriptase (RT) activity and shown to be free of residual inocula. Untreated cells served as a control. Culture supernatants were collected for HIV RT activity assay at days 7 post infection.

HIV RT Assay

HIV RT activity was determined based on the technique [29] with modifications [21].

microRNA Extraction and Quantification

Total cellular RNA, including microRNA (miRNA), was extracted from cells using miRNeasy Mini Kit from QIAGEN (Valencia, CA). Total RNA (1 µg) was reverse-transcribed with miScript Reverse Transcription Kit from QIAGEN. The real-time RT PCR for the quantification of a subset of miRNAs (miRNA-28, miRNA-125b, miRNA-150, miR-198, miRNA-223, and miRNA-382) was carried out with miScript Primer Assays and miScript SYBR Green PCR Kit from QIAGEN as described [30].

RNA Extraction and Real-Time RT-PCR

Total RNA from CBMDM was extracted with Tri-Reagent Sigma (St. Louis, MO). Total RNA (1 µg) was subjected to reverse transcription (Promega, Madison, WI). Real-time PCR was performed with the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) as previously described [31, 32]. The oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) and sequences will be available upon request. The data was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and presented as the change in induction relative to that of untreated control cells.

Statistical Analysis

For comparison of the mean of two groups, statistical significance was assessed by Student's t-test. One-way ANOVA were used for comparison of result between the different groups (multiple comparisons). All graphs were generated and statistical analysis were performed using GraphPad Prism software (San Diego, CA), and all data are presented as mean $\pm$ SD. Statistical significance was defined as $P < 0.05$.

RESULTS

Alcohol Enhances HIV Infection of CBMDM

We first determined the effect of alcohol on HIV infection of CBMDM. Alcohol treatment enhanced the susceptibility of CBMDM to infection of HIV Bal strain, as evidenced by increased RT activity at day 7 after infection (Fig. 1A). In order to determine whether the opioid receptor is involved in alcohol-mediated enhancement of HIV infection, CBMDM were incubated with naltrexone (a pan-opioid receptor antagonist) for 1 h prior to alcohol treatment and HIV infection. Naltrexone completely abrogated the alcohol-mediated enhancement of HIV infection in CBMDM (Fig. 1B).

Alcohol Inhibits Anti-HIV miRNA Expression

Several miRNAs (miRNA-28, 125b, 150, 198, 223, and 382) have been shown to interfere of HIV transcription, suppressing the viral replication in CD4⁺ T cells and macrophages [30, 33, 34]. Thus, we examined whether alcohol has a negative impact on the expression of these anti-HIV miRNAs in CBMDM. We observed a dose-dependent suppression by alcohol of these anti-HIV miRNAs in CBMDM compared with untreated cells (Fig. 2). In addition, we found that the effect of alcohol on anti-HIV miRNA expression in CBMDM was time-dependent (Fig. 3). In order to determine whether the opioid receptor is involved in alcohol-mediated inhibition of anti-HIV expression, CBMDM were incubated with naltrexone (a pan-opioid receptor antagonist) for 1 h prior to alcohol treatment. Naltrexone treatment completely abrogated the alcohol-mediated inhibition of anti-HIV miRNA expression of CBMDM (Fig. 4).

Alcohol Suppresses IFN-Mediated Immunity

Type I IFNs play a crucial role in host cell innate immunity against viral infections, such as HIV. We thus examined whether alcohol can inhibit intracellular IFN gene expression in CBMDM. We found that alcohol treatment considerably suppressed the expression of IFN- α (Fig. 5A)

DISCUSSION

Alcohol consumption and alcohol abuse are prevalent in HIV-positive individuals and have been identified as potential behavioral risk factors for transmission and disease progression. Although some of this effect may be secondary to decreased compliance with HAART regimens, there may be direct effects of alcohol on host defenses. In this study, we examined the impact of alcohol on the HIV infection and the expression of anti-HIV cellular miRNA of CBMDM.

We demonstrated that when exposed to alcohol at clinically relevant concentrations, CBMDM became more prone to HIV infection than unexposed cells, which was evidenced by increased RT activity (Fig. 1). Investigation of the mechanisms of the alcohol action on HIV demonstrated that alcohol inhibited the expression of anti-HIV miRNAs (Figs. 2, 3). We were particularly interested in the miRNA-28, 125b, 150, 223 and 382, as these miRNAs can target highly conserved regions of HIV, present in all HIV clades [37]. These

miRNAs are highly expressed in resting CD4⁺ T cells [33] and monocytes [30]. The levels of these miRNAs correlate with the vulnerability of monocytes and macrophages to HIV infection [30]. Furthermore, we found that alcohol treatment of macrophages could inhibit the expression of miRNA-198, a newly identified anti-HIV miRNA [34] expression (Figs. 2E, 3E). Thus, the demonstration of inhibition of these miRNAs by alcohol provides a probable mechanism for alcohol-mediated enhancement of HIV infection of macrophages. Several previous studies indicate that alcohol interferes with the endogenous opioid system [38–40], signifying that this system is involved in alcohol action. Furthermore, alcohol altered endogenous opioid peptide synthesis and secretion in both the central nervous system [41] and immune system [25]. Alcohol is capable of activating the endogenous opioid system while reinforcing its effects [42–46], because the treatment with naltrexone (pan-opiate antagonist) decreases ethanol consumption by alcoholic subjects [42, 47, 48]. In this study, we demonstrated that alcohol-mediated enhancement of HIV infection and inhibition of anti-HIV miRNA expression were antagonized by naltrexone (Figs. 1B, 4), which has been approved and used in the treatment of opiate and alcohol dependence [49–51]. These data greatly support that the activation of the endogenous opioid system is also implicated in alcohol-mediated enhancement of HIV infection and inhibition of anti-HIV miRNA expression. Our findings that naltrexone (a pan-opiate antagonist) could block the effects of alcohol on HIV and anti-HIV miRNAs provides the biological mechanism for the possible therapeutic benefit of naltrexone in treating HIV-infected pregnant women who use alcohol to reduce HIV perinatal transmission.

In order to further investigate the mechanism(s) responsible for the action of alcohol, we tested the effect of alcohol on the expression of endogenous type I IFN (IFN- α/β) and IRFs, the key regulator of type I IFNs [52]. We demonstrated that alcohol significantly inhibited both IFN- α and IFN- β expression (Fig. 5), which was associated with an intensified susceptibility of CBMDM to HIV infection and enhanced virus infection. These findings support the earlier reports indicating that alcohol suppresses LPS-induced IFN- β production by adult peripheral blood monocytes [53]. Besides recognizing the elements in the IFN promoter to modulate the expression of type I IFN genes selectively, IRFs also regulate the IFN-stimulated response element (ISRE) in some of the IFN-stimulated genes (ISGs), and thereby induce an antiviral state [54, 55]. Among IRFs,

IRF-3, IRF-5 and IRF-7 are the fundamental regulators of type I IFN gene expression induced by viruses [56]. IRF-3/7 is the chief modulator of the type I IFN-dependent immune response, as it not only induces IFN- α expression but additionally activates a number of antiviral ISGs [52, 57, 58]. Hence, the suppression of IRF-7 expression (Fig. 6A) in CBMDM by alcohol treatment elucidates the inhibitory effect of alcohol on type I IFN expression. We also examined the expression of RIG-I, the key sensor that recognizes viral infections and activates IFN signaling pathways [59]. A recent investigation revealed that purified genomic RNA from HIV induced a RIG-I-dependent type I IFN response [60].

Thus, the suppression of RIG-I expression by alcohol (Fig. 6B) should attenuate the intracellular type I IFN-mediated innate immunity against HIV. As described previously, APOBEC3 family members are cellular cytidine deaminases which can hinder the mobility of HIV [35, 36]. In particular, APOBEC3G and APOBEC3F are known to have the ability

to inhibit HIV replication in both CD4⁺ T cells and macrophages [61–64]. APOBEC3G functions by editing the newly synthesized viral DNA or inhibiting the HIV life cycle *via* lethal editing of nascent reverse transcripts [65–67]. APOBEC3F or APOBEC3H also encodes an antiretroviral protein that can be selectively packaged into HIV virions and strongly deters HIV infectivity [68]. Thus, the suppression of the vital members of the APOBEC3 family in macrophages by alcohol (Fig. 7) provides a sound justification for the enhancing effect of alcohol on HIV infection.

In summary, our study provides the compelling evidence that alcohol enhances HIV infection in CBMDM through the suppression of several antiviral factors in the IFN signaling pathway. Although other mechanisms may also be involved in the alcohol action on HIV, the alcohol-mediated HIV enhancement in macrophages may be largely contributed to the suppression of endogenous IFNs and IFN-inducible antiviral genes. Since alcohol ingestion yields profound and adverse effects on the host cell innate immunity that has a critical role in restricting HIV infection in CBMDM, it is likely that maternal alcohol consumption could facilitate vertical transmission of HIV.

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LIST OF ABBREVIATIONS

CBMDM	Cord blood monocyte-derived macrophages
IRF-7	IFN regulatory factor 7
RIG-I	Retinoic acid-inducible gene I
DMEM	Dulbecco's Modified Eagle's Medium
FCS	Fetal calf serum
RT	Reverse transcriptase
miRNA	MicroRNA
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
APOBEC3	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3

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Fig. (1). Alcohol Enhances HIV Infection of CBMDM

(A) Seven-day-cultured CBMDM were incubated with or without alcohol at indicated concentrations for 24h prior to HIV strain (Bal) infection. HIV RT activity was determined at day 7 post-infection. (B) Seven-day-cultured CBMDM were incubated with or without alcohol (40 mM) for 24h prior to HIV Bal strain infection. Naltrexone (10^{-8} M) was added to macrophage cultures for 1h prior to alcohol treatment. HIV RT activity was determined at day 7 post-infection. Cell culture supernatants were collected at indicated time points and subjected to RT assay to detect HIV RT activity. The data shown are the mean $\pm$ SD of triplicate cultures representative of three experiments using cells from three different donors (* $p < 0.05$, $p < 0.01$, alcohol vs control, or alcohol vs alcohol + naltrexone). and IFN- β (Fig. 5B) in CBMDM. We then investigated whether alcohol treatment can modulate the expression of IFN regulatory factor (IRF). Alcohol treatment of CBMDM decreased the expression of IRF7 (Fig. 6A). In addition to IRFs, we also determined the impact of alcohol on retinoic acid inducible gene I (RIG-I), a key intracellular sensor of viral infections. As shown in Fig. (6B), alcohol inhibited RIG-I expression in CBMDM. Furthermore, we tested whether alcohol can inhibit apolipoprotein B mRNA- editing enzyme catalytic polypeptide-

like 3 (APOBEC3) gene expression in macrophages, as several members of APOBEC3 family have the ability to restrict HIV replication [35, 36]. Alcohol-treated CBMDM expressed the lower levels of several members (3G and 3H) of APOBEC3 family than untreated CBMDM (Fig. 7A, B).

Fig. (2). Dose-dependent Effect of Alcohol on Anti-HIV miRNA Expression of CBMDM
Seven-day-cultured CBMDM were incubated in the presence or absence of alcohol at indicated concentrations for the 6h. Total cellular RNA extracted from cell cultures and subjected to realtime RT -PCR for miRNA expression: (A) miR-28, (B) miR-125b, (C) miR-150, (D) miR-223, (E) miR-198, and (F) miR-382. The data shown are the mean $\pm$ SD of triplicate cultures, representative of three experiments using cells from six different donors (*p<0.05, **p<0.01, alcohol vs control).

Fig. (3). Time Course Effect of Alcohol on Anti-HIV miRNA Expression of CBMDM
Seven-day-cultured CBMDM were incubated in the presence or absence of alcohol (40mM) for 3h or 6h. Total cellular RNA extracted from cell cultures and subjected to realtime RT - PCR for miRNA expression: (A) miR-28, (B) miR-125b, (C) miR-150, (D) miR-223, (E) miR-198, and (F) miR-382. The data shown are the mean $\pm$ SD of triplicate cultures, representative of three experiments using cells from three different donors (*p<0.05, **p<0.01, alcohol vs control).

Fig. (4). Opioid Receptor Antagonist Block the Alcohol Action on Anti-HIV miRNA Expression of CBMDM

Seven-day-cultured CBMDM were incubated with or without alcohol at indicated concentrations for 6h. Naltrexone (10^{-8} M) was added to CBMDM cultures for 1h prior to the alcohol treatment. Total cellular RNA were then extracted from cell cultures 6h post-treatment and subjected to realtime RT-PCR for miRNA expression: (A) miR-28, (B) miR-125b, (C) miR-150, (D) miR-223, (E) miR-198, and (F) miR-382. The data shown are the mean $\pm$ SD of triplicate cultures representative of three experiments using cells from three different donors (* $p < 0.05$, ** $p < 0.01$, alcohol vs control, or alcohol vs alcohol + naltrexone).

Fig. (5). Alcohol Inhibits IFN-alpha/beta Expression of CBMDM

Seven-day-cultured CBMDM were incubated in the presence or absence of alcohol at indicated concentrations for 3h or 6h. Total cellular RNA were then extracted from cell cultures and subjected to realtime RT-PCR for IFN-alpha (A) and IFN-beta (B) expression. The data shown are the mean $\pm$ SD of triplicate cultures representative of three experiments using cells from three different donors (* p <0.05, ** p <0.01, alcohol vs control).

Fig. (6). Effect of Alcohol on IRF-7 and RIG-I Expression of CBMDM

Seven-day-cultured CBMDM were cultured with or without alcohol at indicated concentrations for 3h or 6h. Total cellular RNA were then extracted from cell cultures and subjected to realtime RT-PCR for IRF-7 (**A**) and RIG-I (**B**) expression. The data shown are the mean $\pm$ SD of triplicate cultures representative of three experiments using cells from two different donors (* p <0.05, ** p <0.01, alcohol vs control).

Fig. (7). Alcohol Inhibits APOBEC3G/H Expression of CBMDM

Seven-day-cultured CBMDM were incubated in the presence or absence of alcohol at indicated concentrations for 3h or 6h. Total cellular RNA were then extracted from cell cultures and subjected to realtime RT-PCR for APOBEC3G (**A**) and APOBEC3H (**B**) expression. The data shown are the mean $\pm$ SD of triplicate cultures, representative of three experiments using cells from three different donors (* $p < 0.05$, ** $p < 0.01$, alcohol vs control).