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Acrolein enhances epigenetic modifications, FasL expression and hepatocyte toxicity induced by anti-HIV drug Zidovudine

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ABSTRACT

Zidovudine (AZT) remains the mainstay of antiretroviral therapy against HIV in resource-poor countries; however, its use is frequently associated with hepatotoxicity. Not all HIV patients on AZT develop hepatotoxicity, and the determining factors are unclear. Alcohol consumption and cigarette smoking are known risk factors for HIV hepatotoxicity, and both are significant sources of acrolein, a highly reactive and toxic aldehyde. This study examines the potential hepatotoxic interactions between acrolein and AZT. Our data demonstrate that acrolein markedly enhanced AZT-induced transcriptionally permissive histone modifications (H3K9Ac and H3K9Me3) allowing the recruitment of transcription factor NF-kB and RNA polymerase II at the FasL gene promoter, resulting in FasL upregulation and apoptosis in hepatocytes. Notably, the acrolein scavenger, hydralazine prevented these promoter-associated epigenetic changes and inhibited FasL upregulation and apoptosis induced by the combination of AZT and acrolein, as well as AZT alone. Our data strongly suggest that acrolein enhancement of promoter histone modifications and FasL upregulation are major pathogenic mechanisms driving AZT-induced hepatotoxicity. Moreover, these data also indicate the therapeutic potential of hydralazine in mitigating AZT hepatotoxicity.

1. Introduction

Hepatotoxicity is a significant problem for patients with Human Immunodeficiency Virus-1 (HIV) infection who are treated with antiretroviral therapy (ART), and it accounts for 14–18% of HIV patient deaths not associated with AIDS (Price and Thio, 2010). Thus, although ART has greatly reduced the morbidity and mortality in HIV infected patients, its toxic side effects remain a concern and lead to restriction of long-term use or treatment discontinuation (Bica et al., 2001; Nunez and Soriano, 2005; Palella et al., 2006; Woreta et al., 2011). Zidovudine (3-azido-39-deoxythymidine (AZT), a nucleoside reverse transcriptase inhibitor (NRTI), was the first FDA-approved drug for HIV patients, and continues to be the mainstay of HIV-ART in developing countries (Fang and Beland, 2009; Kalyesubula et al., 2011). Because of its efficacy, AZT is used alone or in combination for prevention of perinatal transmission and combined antepartum, intrapartum, and infant antiretroviral prophylaxis in resource-poor settings, as well as in the United States (Senise et al., 2011; Sturt et al., 2010; website, 2016). Hence, AZT remains a relevant constituent of anti-HIV therapy worldwide. However, long-term use of AZT is associated with significant hematologic disorders, cardiomyopathy and hepatotoxicity (Chariot et al., 1999; Desai et al., 2012; Szabados et al., 1999). With regard to hepatotoxicity, AZT is known to cause mitochondrial dysfunction and apoptosis, however, the detailed mechanisms are not completely understood (Acosta and Grimsley, 1999; Banerjee et al., 2013; Chariot et al., 1999; de la Asuncion et al., 1999; Freiman et al., 1993).

Notably, not all HIV-infected patients on ART develop liver toxicity; up to 20% have elevated liver enzymes and only 2–10% develop severe hepatic injury (Acosta and Grimsley, 1999; Johnson et al., 2001). The factors that determine susceptibility to ART hepatotoxicity are not clearly established; however environmental and dietary factors have the potential to contribute to liver injury. In this regard, cigarette smoking and alcohol consumption are the two common contributing risk factors that occur frequently in HIV infected individuals and are known to adversely influence their health (Lloyd-Richardson et al., 2008; Miguez et al., 2003). The rate of smoking is 2–3 times higher among HIV-positive adults compared to the general public, with high usage of 16–23 cigarettes per day (Benard et al., 2007; Lloyd-Richardson et al., 2008).

Abbreviations: AZT, azidothymidine or Zidovudine; ACR, acrolein; ART, antiretroviral therapy; ChIP, chromatin immunoprecipitation; FasL, Fas ligand; H3K9Ac, histone 3 lysine 9 acetylation; H3K4Me3, histone 3 lysine 4 trimethylation; RNA Pol II, RNA polymerase II; TF, transcription factor; UTR, translational region; TSR, transcription start region.

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Similarly, alcohol use is more frequent among HIV-infected persons with 50% reporting regular alcohol use, and half as many reporting excessive/hazardous alcohol consumption (Galvan et al., 2002). Both alcohol and smoking are independently known to decrease cellular antioxidant status, enhance oxidative stress and increase cellular lipid peroxidation (LPO) (Papadopoulou and Bloomer, 2007), and increased lipid peroxidation is documented in HIV-infected individuals (Teto et al., 2013; Vassimon et al., 2010). Acrolein is one of the most toxic and reactive aldehyde byproducts of lipid peroxidation (Moghe et al., 2015), and is a major aldehyde component of cigarette smoke (Cerami et al., 1997; Hristova et al., 2012; Muscat et al., 1991). Thus, the two common comorbidities in HIV are known sources of acrolein. Reported or calculated levels of acrolein exposure and/or generation vary widely and it is difficult to extrapolate the acrolein concentrations that may be relevant in the liver. Studies have shown that higher acrolein metabolite levels ranging from approximately 30 μM to 180 μM are detected in body fluids under pathological conditions (Carmella et al., 2007; Eiserich et al., 1995; Calingasan et al., 1999; Lovell and Markesbery, 2001; Sakata et al., 2003; Satoh et al., 1999; Tsukahara et al., 2002). Notably, our earlier studies have examined acrolein toxicity (5 μM to 100 μM) in hepatocytes, and shown that acrolein has multiple adverse effects in hepatocytes (such as GSH depletion, mitochondrial dysfunction and ER stress) which contribute to its toxicity (Mohammad et al., 2012). However, the interactions of acrolein and ART have not been studied, and it is unclear whether acrolein exposure predisposes to or potentiates ART hepatotoxicity.

The role of the Fas-FasL signaling pathway in the apoptotic death of hepatocytes has been demonstrated in several forms of liver disease, particularly drug-induced liver injury (Feldstein et al., 2003; Malhi et al., 2010; Pianko et al., 2001). Typically, hepatocytes express high levels of Fas receptor and respond to FasL signals from activated immune cells; however, under certain conditions, hepatocytes also express high levels of FasL, leading to fratricide and liver injury (Galle et al., 1995). With regard to HIV-positive individuals, increased expression of FasL is seen in cardiomyocytes, which contributes to AZT-Induced cardiomyopathy (Purevjav et al., 2007). However, the role of FasL expression and apoptotic death in AZT induced hepatotoxicity has not been examined. We and others have shown that promoter-associated epigenetic modifications are known to regulate FasL gene expression (Castellano et al., 2006; Ghare et al., 2014; Holtz-Heppelmann et al., 1998; Li-Weber and Krammer, 2003). Hence, the present study examines the potential hepatotoxic interactions between acrolein and the ART drug, AZT. Specifically, we investigated the effect of acrolein and AZT on the epigenetic regulation of FasL, and consequent FasL-mediated apoptotic death of hepatocytes.

2. Materials and methods

2.1. Cell culture

HepG2 cells (clone E6-1) obtained from ATCC (ATCC, Rockville, MD) were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 U/ml penicillin and 10 μg/ml streptomycin. Cells were maintained in an incubator at 37 °C with humidified 5% CO_2 and used in passages 3–7. Cells were plated (at 0.25 million cells per ml) and incubated overnight in media containing 10% FBS. In all experiments, treatments were done in serum-free media.

Primary rat hepatocytes were obtained from TRL (Triangle Research Laboratory, NC) and maintained in accordance with company instructions. For experiments, cells were plated (at 0.25 million cells per ml) on non-collagen coated plates (to maintain ACR reactivity) and kept in an incubator at 37 °C with humidified 5% CO_2 overnight. Treatments for all experiments were done in serum free Dulbecco's modified eagle medium (DMEM) supplemented with 10 U/ml penicillin and 10 μg/ml streptomycin.

2.2. Reagents

AZT, acrolein and hydralazine were purchased from Sigma-Aldrich (St. Louis, MO).

Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). DMEM, penicillin, streptomycin and TRIzol® were obtained from Invitrogen (Carlsbad, CA). Caspase-8 inhibitor Z-IETD-FMK was purchased from R&D Systems, Inc. (Minneapolis, MN). Antibodies for FasL, Caspase 8, Caspase 3, and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Treatments

AZT and hydralazine stock solutions were made in water and stored as per the company instructions. Acrolein was made in serum free media and immediately used for the experimental treatments. Caspase-8 inhibitor was dissolved in DMSO to yield a concentrated stock solution, such that final concentration of DMSO in the treatment will be less than 0.1%. Treatment with DMSO alone served as a negative solvent control (data not shown).

The final concentrations used in the study were 100 μM and 250 μM for AZT and 12.5 μM and 25 μM for acrolein. These concentrations were selected based upon the results from preliminary dose response experiments (data not shown) and published literature (Fang and Beland, 2009; Fang et al., 2014; Mohammad et al., 2012; Sun et al., 2014).

2.4. Cell viability-MTT assay

Cell survival/cell death was measured in treated cells by the MTT (3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) assay as described previously (Mohammad et al., 2012).

2.5. DNA fragmentation ELISA

DNA fragmentation was measured using a Cell Death Detection ELISA kit (Roche Applied Sciences, Indianapolis, IN) in accordance with manufacturer's instructions (Mohammad et al., 2012).

2.6. RNA isolation and real time PCR analysis

Total RNA was isolated from cells using TRIzol® (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. For real-time PCR, the first-strand cDNA was synthesized using qScriptTM cDNA SuperMix (Quanta Biosciences, Inc., Gaithersburg, MD) using 100 ng of total RNA. The RT conditions were 10 min at 25 °C, 30 min at 48 °C and 5 min at 85 °C. Real time PCR was performed with an ABI prism 7500 sequence detection system and PerfeCtaTM SYBR Green FastMixTM, Low ROX reagents (Quanta Biosciences, Inc.). Each sample was run in duplicate or triplicate reactions as technical replicates. Reverse transcriptase polymerase chain reaction (RT-PCR) assays were used to assess mRNA levels. Several housekeeping genes (β-actin, TATA binding protein, and 18s rRNA) were initially used for normalization and optimization, and no significant differences were seen. For the manuscript, only data normalized with 18s rRNA is shown. FasL and 18s rRNA specific primers were designed in the lab and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Primers used were as follows:

FasL-FP 5′-TCTACCAGCCAGATGCACAC-3′, FasL-RP 5′-CAGAGGCATGGACCTTGAGT-3′. 18s-FP 5′-CTCAACACGGGAAACCTCAC-3′, 18s-RP 5′-CGCTCCACCAACTAAGAAC-3′.

The parameter threshold cycle (C_t) was defined as the fraction cycle number at which the fluorescence passed the threshold. The gene expression was analyzed by relative quantification using $2-\Delta\Delta Ct$ method by normalizing with 18s rRNA and data is presented as fold change over UT which is set at 1.