Research Article

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Alcohol stimulates macrophage activation through caspase-dependent hepatocyte derived release of CD40L containing extracellular vesicles

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Background & Aims: The mechanisms by which hepatocyte exposure to alcohol activates inflammatory cells such as macrophages in alcoholic liver disease (ALD) are unclear. The role of released nano-sized membrane vesicles, termed extracellular vesicles (EV), in cell-to-cell communication has become increasingly recognized. We tested the hypothesis that hepatocytes exposed to alcohol may increase EV release to elicit macrophage activation.

Methods: Primary hepatocytes or HepG2 hepatocyte cell lines overexpressing ethanol-metabolizing enzymes alcohol dehydrogenase ($HepG2^{ADH}$) or cytochrome P450 2E1 ($HepG2^{Cyp2E1}$) were treated with ethanol and EV release was quantified with nanoparticle tracking analysis. EV mediated macrophage activation was monitored by analysing inflammatory cytokines and macrophage associated mRNA expression, immunohistochemistry, biochemical serum alanine aminotransferase and triglycerides analysis in our *in vitro* macrophage activation and *in vivo* murine ethanol feeding studies.

Results: Ethanol significantly increased EV release by 3.3-fold from $HepG2^{Cyp2E1}$ cells and was associated with activation of caspase-3. Blockade of caspase activation with pharmacological or genetic approaches abrogated alcohol-induced EV release. EV stimulated macrophage activation and inflammatory cytokine induction. An unbiased microarray-based approach and antibody neutralization experiments demonstrated a critical role of CD40 ligand (CD40L) in EV mediated macrophage activation. *In vivo*, wild-type mice receiving a pan-caspase, Rho kinase inhibitor or with genetic deletion of CD40 ($CD40^{-/-}$) or the caspase-activating TRAIL receptor ($TR^{-/-}$), were protected from alcohol-induced injury and associated macrophage infiltration. Moreover, serum from patients with alcoholic hepatitis showed increased levels of CD40L enriched EV.

Conclusion: In conclusion, hepatocytes release CD40L containing EV in a caspase-dependent manner in response to alcohol exposure which promotes macrophage activation, contributing to inflammation in ALD.

Keywords: Alcoholic hepatitis; Caspase inhibitor; Liver; Inflammation; TNF-α; CD40L; Hepatocyte; Macrophage; Cyp2E1; Alcohol; Exosomes.

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Introduction

Alcoholic liver disease (ALD) is an important cause of preventable death with a potential for 30–40% 1-month mortality among those with alcoholic hepatitis (AH) [1]. Current concepts in ALD pathogenesis include enhanced ethanol induced intestinal permeability leading to hepatic delivery of gut derived lipopolysaccharide (LPS) [2,3] as well as direct hepatic effects of ethanol on hepatocytes [4]. While alcohol is metabolized primarily by hepatocyte alcohol dehydrogenase [2], chronic and/or high dose alcohol exposure lead to metabolism by the hepatic microsomal mono-oxygenases, particularly cytochrome P4502E1 [5]. Alcohol metabolism contributes to liver injury via acetaldehyde generation and production of reactive oxygen species (ROS) [2]. However, there is lack of information pertaining to the signals that connect hepatocyte injury with macrophage activation in this process.

Recent studies show that cells undergoing stress or injury may release nano-sized vesicles such as microparticles or exosomes, referred to here as extracellular vesicles (EV) [6,7]. These EV contain molecules that may transfer signals from one cell to another through contents that include mRNA, miRNA, lipids, DNA or proteins. This mechanism could conceivably link epithelial cell injury with its associated inflammatory response. However, the role of EV in alcohol-induced liver injury has not been expansively explored previously.

We sought to identify mechanisms by which hepatocyte exposure to alcohol could lead to macrophage activation by addressing several specific questions: 1) Does hepatocyte exposure to alcohol lead to EV production? 2) If so, then what mechanisms mediate EV release? 3) Does EV release lead to macrophage activation and inflammation? 4) If so, then how?

We show here that upon ethanol exposure and metabolism, hepatocytes release EV. Both *in vitro* and *in vivo* studies show that this process requires TRAIL receptor (TR) and caspase-3 activation. We further show that CD40L contained within EV contributes to macrophage activation associated with alcohol-induced injury. Thus, the studies mechanistically extend existing paradigms relating to alcohol-induced liver inflammation by showing a role for caspase-dependent EV production and CD40L that mediate pathobiological paracrine signaling.

Materials and methods

Cell culture

HCC cell lines HepG2, HepG2^{ADH} and HepG2^{Cyp2E1} were routinely cultured in EV reduced DMEM, supplemented with 10% FBS. The HepG2^{Cyp2E1} cells were kind gift from Dr. A. Cederbaum, (Mount Sinai School of Medicine, New York) [5] and Hep^{ADH} cells were a kind gift from Dr. Mark McNiven (Mayo Clinic) [8]. Murine primary hepatocytes were isolated as described earlier [9]. Cells were cultured in reduced EV medium and stimulated with ethanol (0–100 mM) as indicated in individual experiments. For *in vitro* studies, a pan-caspase inhibitor (IDN-7314, 20 μM), diallyl sulphide (DAS; 100 μM), cyanamide (10 μmol/L), or a Rho kinase inhibitor (fasudil; 50 μM) were added prior to addition of ethanol.

Isolation of EV

EV were isolated as described earlier [10]. The pellets obtained were resuspended in a large volume of PBS and washed three times by centrifugation for 2 h at 110,000 g at 4 °C. EV numbers were quantified and characterized using nanoparticle tracking analysis (NTA) as described [11]; (NS300, Malvern Instruments, Malvern, UK). Reduced EV media was prepared by ultracentrifugation of 20% FBS containing DMEM for 2 h in a SW32Ti Rotor at 100,000 g at 4 °C in an Optima XPN-80 ultracentrifuge. Murine serum EV were isolated using TLA100 rotor in an Optima TLX Ultracentrifuge. Reduced EV medium was further used for culture at final 10% FBS concentration.

Caspase assay

Caspase activation in response to ethanol was monitored using methods described earlier, using a Synergy H1 multi plate reader (Bio-Tek Instruments) [12].

Adhesion assay

Cell adhesion was assessed by a 96-well format CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay reagent (Promega) as per the manufacturer's instruction and as described earlier [13]. The cells were treated with 1×10^{11} amounts of EV or EV free culture supernatant as a control [14].

Quantitative real-time PCR (RT-PCR)

The macrophages were treated with EV or EV free medium. Following treatment, total RNA was isolated from cells or whole livers using TRIzol (Invitrogen, Carlsbad, CA) and RNeasy kits (Qiagen) with modifications as described [15,16]. RT-PCR was performed using ABI7500 Real-Time PCR Detection System (Applied Biosystems, Foster City, CA) and the C_T values were normalized to GAPDH or β-actin. The primers used for real-time PCR are listed in Supplementary Table 1.

Chemokine/cytokine arrays

EV isolated from control or ethanol treated HepG2^{Cyp2E1} cells and AH patient samples were subjected to human cytokine and chemokine antibody-based arrays (ARY05; R&D Systems, USA) according to manufacturer's instructions, and quantitated using ImageJ-software (NIH).

Mouse model of chronic plus single binge ethanol consumption

All animal experiments followed protocols approved by Mayo Clinic Institutional Animal Care and Use Committee. For *in vivo* ethanol feeding studies, chronic-binge ethanol feeding (Gao-Binge model), with 12–13 week old wild-type (WT), TR$^{-/-}$, *CD40*$^{-/-}$ C57 / BL6 background female mice was used [17]. The WT & *CD40*$^{-/-}$ mice were purchased from Jackson Laboratories, and the TR$^{-/-}$ mice were kindly provided by Wafik S El-Deiry [18,19]. WT mice received control, ethanol with daily oral administration of compound (IDN-7314; 5 mg/kg in 0.5% carboxy-methyl-cellulose), Rho kinase inhibitor (ROCK1); 50 mg/kg or vehicle alone. The TR$^{-/-}$ or *CD40*$^{-/-}$ mice were similarly subjected to pair fed control or ethanol diet regimen (n = 5–10 per group).

For further details on methodology, please see the Supplementary Methods.

Results

Hepatocytes release EV in response to alcohol exposure

Recent work has shown that hepatocytes can generate EV in response to diverse stimuli [20]. As an initial step to explore this

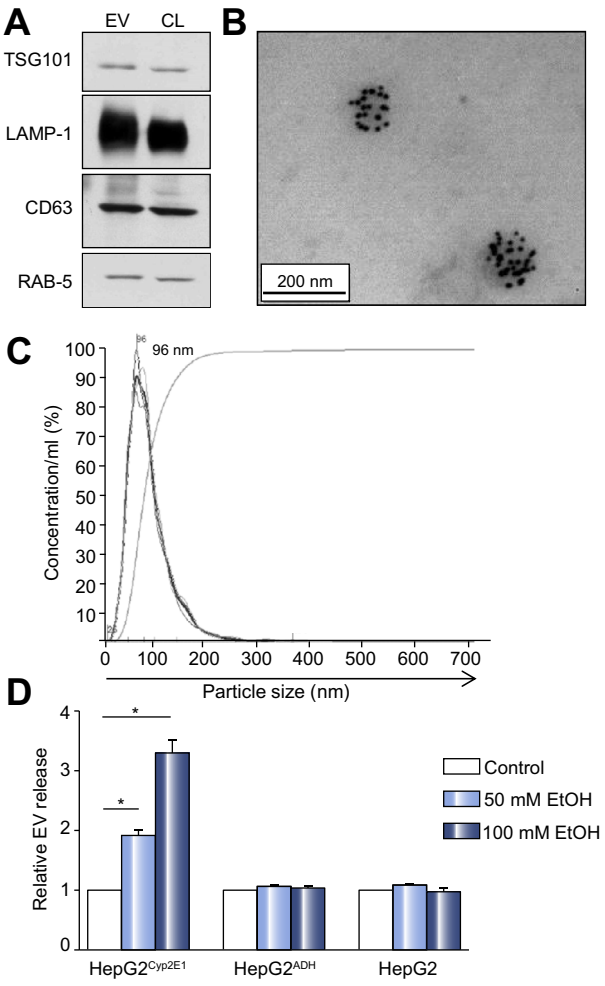

Fig. 1. Characterization and quantification of EV produced by hepatocytes in response to alcohol treatment. (A) Western blot analyses for membrane vesicle markers from EV or CL from HePG2^{Cyp2E1} (30 μg) were analysed in parallel. Four different membrane vesicles associated markers (TSG101, LAMP-1, CD63 and RAB5) are shown. (B) Representative image of EM analysis shows morphology size and presence of EV associated marker, TSG101. (C) Representative plot showing size distribution of EV was carried out using NTA. (D) Quantification of EV was done using NTA. EV release was significantly increased in HepG2^{Cyp2E1} cells upon ethanol stimulation (50,100 mM), whereas no increase in EV release was observed in HepG2^{ADH} or in control HepG2 cells stimulated with ethanol (n = 3, $^{*}p$ <0.05).