Research Article

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Alcohol directly stimulates epigenetic modifications in hepatic stellate cells

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Background & Aims: Alcohol is a primary cause of liver disease and an important co-morbidity factor in other causes of liver disease. A common feature of progressive liver disease is fibrosis, which results from the net deposition of fibril-forming extracellular matrix (ECM). The hepatic stellate cell (HSC) is widely considered to be the major cellular source of fibrotic ECM. We determined if HSCs are responsive to direct stimulation by alcohol.

Methods: HSCs undergoing transdifferentiation were incubated with ethanol and expression of fibrogenic genes and epigenetic regulators was measured. Mechanisms responsible for recorded changes were investigated using ChIP-Seq and bioinformatics analysis. Ethanol induced changes were confirmed using HSCs isolated from a mouse alcohol model and from ALD patient's liver and through precision cut liver slices.

Results: HSCs responded to ethanol exposure by increasing pro-fibrogenic and ECM gene expression including elastin. Ethanol induced an altered expression of multiple epigenetic regulators, indicative of a potential to modulate chromatin structure during HSC transdifferentiation. MLL1, a histone 3 lysine 4 (H3K4) methyltransferase, was induced by ethanol and recruited to the elastin gene promoter where it was associated with enriched H3K4me3, a mark of active chromatin. Chromatin immunoprecipitation sequencing (ChIPseq) revealed that ethanol has broad effects on the HSC epigenome and identified 41 gene loci at which both MML1 and its H3K4me3 mark were enriched in response to ethanol.

Conclusions: Ethanol directly influences HSC transdifferentiation by stimulating global changes in chromatin structure, resulting in the increased expression of ECM proteins. The ability of alcohol to remodel the epigenome during HSC transdifferentiation provides mechanisms for it to act as a co-morbidity factor in liver disease.
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Introduction

Chronic alcohol consumption is both a direct cause of liver disease as well as a major co-morbidity factor in the progression of liver disease, resulting from other primary causes such as viral hepatitis [1,2]. Mechanisms explaining the hepatotoxicity of alcohol are beginning to be understood and help to explain its impact on liver disease. Alcohol and its metabolites, in particular reactive oxygen species (ROS), such as the hydroxyethyl radical and nitric oxide are a major cause of hepatocellular damage [3]. Furthermore these metabolites can induce hepatic inflammation, which via cytokines such as TNFα are an important indirect cause of alcohol-induced hepatocyte damage and death. In addition, ROS-mediated lipid peroxidation has been proposed to cause damage to hepatocyte proteins, which can drive antigenic responses that perpetuate inflammation and liver damage [4].

Hepatocellular damage and inflammation stimulate the transdifferentiation of resident perisinusoidal HSCs into α-smooth muscle actin (αSMA)-positive myofibroblasts. These so-called "activated" HSCs (aHSCs) are the major hepatocellular source of fibrotic ECM proteins and promote the net deposition of fibrotic ECM in chronic liver disease [5]. The molecular processes that promote the progression of liver disease to severe fibrosis remain poorly defined, however, the continued production of ECM proteins by aHSCs is a major contributory factor. In addition, aHSCs secrete proteins that promote cross-linking, maturation and insolubility of the fibrotic ECM, such as elastin. Elastin is a target for cross-linking, catalysed by lysyl-oxidase or tissue transglutaminase [6], and its accumulation in the fibrotic ECM limits its

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Abbreviations: ALD, alcoholic liver disease; ASH1, absent, small or homeotic disc 1; ChIP, chromatin immunoprecipitation; ECM, extra cellular matrix; H3K4, lysine 4 histone 3; HSC, hepatic stellate cell; MeCP2, methyl-CpG binding protein 2; MLL1, myeloid/lymphoid, or mixed-lineage, leukemia; MNase, mononuclease; NHL, normal human liver; PPARγ, peroxisome proliferator-activated receptor-γ; qHSC, quiescent hepatic stellate cell; rHSC, rat hepatic stellate cell; qPCR, quantitative polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; αSMA, alpha smooth muscle actin; TGF-β1, transforming growth factor beta 1; TIMP-1, tissue inhibitor of metalloproteinases 1.

potential for degradation and impacts on the degree of reversibility of the fibrotic tissue. Hence, factors that stimulate the expression of ECM maturation proteins, such as elastin, are likely to determine fibrosis progression in liver disease.

Recent studies from our laboratory and other investigators have revealed the importance of epigenetic signalling events in HSC transdifferentiation and fibrogenesis. Experimental manipulation of epigenetic signatures, such as DNA methylation, histone acetylation/methylation, and the activities of proteins that either annotate or interpret these epigenetic marks, can have profound effects on the HSC phenotype [7]. The concept that alcohol can stimulate epigenetic changes in liver tissue is already well established. Ethanol impairs the normal metabolism of methionine and in turn affects the availability of methyl groups in form of S-adenosylmethionine (SAMe) for DNA and histone methylation [8]. Kendrick and colleagues showed that ethanol or acetate reduced histone deacetylase activity and upregulated expression of acetyl-CoA synthetases, resulting in increases in histone acetylation and transcriptional activity at inflammatory genes [9]. Less is understood concerning the potential for alcohol to influence the HSC epigenome and expression of ECM proteins.

Here, we investigated the impact of alcohol on the expression of epigenetic regulators during HSC transdifferentiation and report that histone-modifying enzymes, such as the H3K4 methyltransferase MLL1, are upregulated in HSC exposed to ethanol and lead to the altered expression of profibrogenic genes including elastin. We provide combinatorial chromatin immunoprecipitation sequencing (ChIPseq) analysis on H3K4me3 and MLL1 signature in ethanol-stimulated HSCs that reveals widespread changes in chromatin structure, confirming a profound genome-wide effect of alcohol on HSC transdifferentiation. We suggest that our findings provide new insights into the mechanisms by which alcohol acts as a co-morbidity factor and a stimulator of fibrosis in chronic liver disease.

Materials and methods

Ethics

The authors hold appropriate licences for animal experiments, which were issued/approved by the local ethical committee and UK Home Office.

Human subjects

Use of human tissue was approved by the Newcastle and North Tyneside Local Research Ethics committee (approval number H10/H0906/41). All samples were collected and used subject to patient's written consent.

Cell isolation and culture

All rats were purchased from Charles River, UK. Rat hepatic stellate cells (rHSCs) were isolated from normal livers of 350 g Sprague-Dawley rats by sequential perfusion with collagenase and pronase, followed by discontinuous density centrifugation in 11.5% OptiPrep (Sigma-Aldrich, Gillingham, Dorset, UK). Cells were cultured on plastic dishes in Dulbecco's modified Eagle's medium, supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 16% foetal calf serum and maintained in an incubator at 37 °C at an atmosphere of 5% CO_2. HSCs were treated at day 1, 2, and 10 of culture for 24 h and 48 h with 86 mM ethanol (dose equivalent to heavy drinking in humans) [10]. In order to create an atmosphere saturated with ethanol and prevent evaporation from the culture media, 0.5% ethanol in water was added to the incubator 24 h before the treatment. Acetate treatment day 2 cultures of HSCs were treated with 1 mM sodium acetate, which is an acetate concentration found in a person that is metabolizing ethanol at a concentration of 86 mM [9].

Animal model

Male *Col1a1-GFP* mice (12 wk old) were fed *ad libitum* for 2 weeks (wk) with a liquid "Western diet" (WD) high in cholesterol (1% or 0.5%) and saturated fat (27% Cal or 22% Cal as lard). Then, the diet was switched to an ethanol-containing Western diet for 8 weeks. Ethanol intake was gradually increased from 1% (w/v) on day 1 to 4.5% (w/v) on day 12 until the end of the end of 8 weeks feeding. From the second week of ethanol feeding, a weekly binge dose of alcohol was given via a stomach tube and repeated 7 times. The binge dose was gradually increased from 3.5 g/kg to 4.5 g/kg. For control mice, age and gender matched *Col1a1-GFP* mice were fed regular chow.

HSC isolation from the animal model

A non-parenchymal liver cell fraction was collected by pronase-collagenase perfusion and gradient ultracentrifugation. GFP fluorescence was excited at 488 nm by an argon laser and measured through a 530 nm filter and the vitamin A UV fluorescence was excited at 350 nm by a krypton laser and measured through a 350 nm filter. UV^-/GFP^+ cells (aHSCs) were collected in tubes containing the medium with DNAse.

Cross-linked chromatin immunoprecipitation (XChIP) assay

Chromatin immunoprecipitation (ChIP) assays for MLL1 and H3K4me3 binding were carried out by using 50 μg cross-linked chromatin, prepared by fixing cells in 1% formaldehyde, then lysed by sonicating cells for 5 min in Diagenode Bioruptor. Precleared chromatin was incubated with 5 μg anti MLL1 or H3K4me3 antibodies, the complexes were precipitated, washed and eluted. Crosslinks were reversed and genomic DNA was purified. Each PCR reaction was performed in duplicate and the analysis was repeated at least three times from independent ChIP experiments. A signal intensity value for each sample was calculated from the average of the experiments. Average values of eluates were normalized to average values of the control antibody sample and expressed as fold enrichment above background (i.e. control antibody). Quantitative PCR amplification was carried out using primers listed in Supplementary Tables 2 and 3.

ChIP-seq and bioinformatic analysis

ChIP assays were carried out as above. 500 ng of genomic DNA was used to construct a library according to the TruSeq DNA (LS) protocol by Illumina. The DNA fragments were selected for 550 bp insert size. In order to increase the final concentration of the library, ten cycles of PCR reaction were performed. ChIP-seq results were validated by standard ChIP assays and quantitative PCR was performed using primers in Supplementary Table 3. ChIPseq data were analysed using the Model-based Analysis of ChIP-Seq (MACS) software, with Bowtie 2 alignments against the annotated rat genome from Ensembl (Rnor_5.0), and compared against the control for each immunoprecipitation. Peaks were called significant with a *p* value of 1e-07. The resulting peak list was visualised using IGV browser (http://www.broadinstitute.org/igv/home).

Immunohistochemistry, SDS-PAGE and immunoblotting, quantitative PCR (RTqPCR analysis), and micrococcal nuclease digestion are in the Supplementary Materials and methods section.

Results

Ethanol promotes HSC transdifferentiation and stimulates the expression of histone modifying enzymes in transdifferentiating HSCs

Culture-induced activation of primary rat HSCs is associated with time-dependent increases in the transcriptional activity and expression of ECM genes including type I and III collagen, tropoelastin (elastin) and tissue inhibitor of metalloproteinases 1 (*TIMP1*) (Supplementary Fig. 1). Freshly isolated (day 1) HSCs expressed low or undetectable levels of transcript for these genes, which were subsequently induced to detectable levels by day 3 of culture, and continued to rise in expression with further length of culture as cells acquired their myofibroblastic phenotype. To determine

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