Research Article

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Ubiquitin C-terminal hydrolase 1: A novel functional marker for liver myofibroblasts and a therapeutic target in chronic liver disease

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Background & Aims: Ubiquitination is a reversible protein modification involved in the major cellular processes that define cell phenotype and behaviour. Ubiquitin modifications are removed by a large family of proteases named deubiquitinases. The role of deubiquitinases in hepatic stellate cell (HSC) activation and their contribution to fibrogenesis are poorly defined. We have identified that the deubiquitinase ubiquitin C-terminal hydrolase 1 (UCHL1) is highly induced following HSC activation, determined its function in activated HSC and its potential as a therapeutic target for fibrosis.

Methods: Deubiquitinase expression was determined in day 0 and day 10 HSC. Increased UCHL1 expression was confirmed in human HSC and in an alcoholic liver disease (ALD) patient liver. The importance of UCHL1 in hepatic fibrosis was investigated in CCl_4 and bile duct ligation injured mice using a pharmacological inhibitor (LDN 57444). The effects of UCHL1 inhibition on HSC proliferation were confirmed by Western blot and 3H thymidine incorporation.

Results: Here we report that pharmacological inhibition of UCHL1 blocks progression of established fibrosis in CCl_4 injured mice. UCHL1 siRNA knockdown, LDN 57444 treatment, or HSC isolated from *UCHL1*$^{-/-}$ mice show attenuated proliferation in response to the mitogen, platelet-derived growth factor. Additionally, we observed changes in the phosphorylation of the cell cycle regulator retinoblastoma protein (Rb) in the absence of UCHL1 highlighting a potential mechanism for the reduced proliferative response.

Conclusions: UCHL1 expression is highly upregulated upon HSC activation and is involved in the regulation of HSC proliferation. This study highlights therapeutic opportunities for pharmacological targeting of UCHL1 in chronic liver disease.

Keywords: Deubiquitinase; UCHL1; HSC; Liver fibrosis; LDN57444.

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Abbreviations: HSC, hepatic stellate cells; UCHL1, ubiquitin c-terminal hydrolase; Ub, ubiquitin; DUB, deubiquitinase; USP, ubiquitin specific protease; IPF, idiopathic pulmonary fibrosis; CCl_4, carbon tetrachloride; BDL, bile duct ligation; qHSC, quiescent hepatic stellate cells; aHSC, activated hepatic stellate cells; αSMA, alpha smooth muscle actin; ALD, alcoholic liver disease; PDGFBB, platelet-derived growth factor BB; CDK, cyclin-dependent kinase; Rb, retinoblastoma protein.

Introduction

Activation or transdifferentiation of quiescent hepatic stellate cells (HSC) to a myofibroblast state is central to the fibrogenic process in acute hepatic wound-repair and chronic liver disease [1,2]. HSC transdifferentiation is an epigenetically regulated process that induces genome-wide changes in gene expression that enable the cell to adopt its profibrogenic functions including proliferation, migration and the expression and secretion of large quantities of extracellular matrix proteins. To acquire the capability for these functions the transdifferentiated HSC must also undergo fundamental alterations in protein turnover and in the post-translational regulatory mechanisms that control the localisation and function of proteins. The major post-translational processes that control cell phenotype and behaviour include the addition of small chemical groups (e.g. by phosphorylation, acetylation, methylation and hydroxylation), alterations in the chemical nature of amino acids (e.g. by citrullination), addition of functional molecules (e.g. lipids or sugars) or the covalent linkage of proteins to other protein or peptide modules (e.g. by ubiquitination, sumoylation, and neddylation) [3]. While there have been many studies describing the role of protein phosphorylation in HSC, principally concerning signal transduction events implicated in fibrogenesis, there has been relatively little attention to the contributions of other forms of protein modifications towards HSC activation and fibrosis.

Ubiquitin (Ub) is a small and highly conserved protein comprised of 76 amino acids that is expressed throughout the eukaryotic kingdom. The addition of Ub to proteins is controlled by the coordinated activity of three types of enzymes; Ub activating enzyme (E1), Ub conjugating enzyme (E2) and Ub ligase (E3). Ub is conjugated either as a monomer or end-to-end polymer

chain, but more commonly as a branched polymer where seven different lysine (K) residues (K6, K11, K27, K29, K33, K48, and K63) within the Ub peptide can serve as points of Ub-Ub conjugation. The best-characterised poly-Ub chains are the K48-linked polymers, which target their associated proteins for processing or degradation by the 26S proteasome [4]. By contrast, K63-linked chains avoid the 26S proteasome and instead are more akin to phosphorylation in that they regulate a wide variety of signalling events such as activation of NF-κB [5]. Ubiquitination is a reversible modification, important for termination of signalling events, editing of Ub-protein modifications and recycling of Ub [6]. Deubiquitination is catalysed by a large family of over 80 different deubiquitinases (DUBs), which can be subdivided into five distinct super-families, the ubiquitin specific proteases (USPs), the ubiquitin C-terminal hydrolases (UCHs), the Machado-Josephin domain proteases (MJDs), the ovarian tumour proteases (OTU) and the JAB1/MPN/Mov34 (JAMM) domain metalloproteinases [7]. DUB specificity is also determined by cellular location, specific binding interactions and the type of ubiquitin chain linkage and removal of these ubiquitin adducts regulates either the proteins stability or its activity [8]. Noteworthy is that several DUBs have been identified with polymorphisms that are linked to a range of human diseases including cancers and neurological disorders [9–12].

Given the important regulatory role of DUBs in ubiquitination we hypothesised that HSC transdifferentiation will be associated with stable alterations in the expression of the DUBs and will be associated with specific functional requirements of the activated HSC. By testing this hypothesis we hoped to identify one or more DUBs that may be targeted to attenuate the fibrogenic activities of HSC. Here, we present data demonstrating that ubiquitin C-terminal hydrolase 1 (UCHL1 or PGP9.5) is highly induced with HSC activation and plays a role as a regulator of HSC proliferation. Moreover, we show that pharmacological inhibition of UCHL1 blocks HSC proliferation and when administered *in vivo* acts in a therapeutic way to block progression of established fibrosis despite continued liver injury.

Materials and methods

Human samples

Alcoholic liver disease (ALD) and human control liver, normal and diseased lung (idiopathic pulmonary fibrosis, IPF) and kidney tissue samples for histology were taken under full ethical approval with patient consent (REC references 10/H0906/41, 11/NE/0291, and 13/EM/0311).

Animals

A description of the *UCHL1*$^{-/-}$ mice including details of the knockout strategy appear in Coulombe *et al.*, [13]. Authors hold appropriate licences for work relating to all experiments and animal procedures were approved by local ethical review committee and the UK Home Office.

Chronic carbon tetrachloride (CCl_4) liver injury model

Wild-type (WT) male mice (25–30 g) were intraperitoneal injected twice weekly for 8 weeks with CCl_4 at 2 μl/g body weight (CCl_4:olive oil at 1:3[vol:vol]) or olive oil vehicle. From week 4 mice received an additional bi-weekly injection intraperitoneal of UCHL1 inhibitor LDN 57444 (0.4 mg/kg) or vehicle (DMSO). 24 h after the final CCl_4 injection mice were humanely killed and liver and serum samples prepared.

Bile duct ligation

Bile duct ligation (BDL) was performed as previously described under buprenorphine pain relief [14]. Mice were allowed to develop cholestatic disease and fibrosis over a period of 14 days. Alzet osmotic minipumps were implanted subcutaneously at day 0 delivering (0.4 mg/kg/day) LDN 57444 or (DMSO 50%, PEG 50%) vehicle control. Animals were humanely killed and liver and serum samples prepared.

Isolation of primary HSC

Primary human HSCs were isolated from normal margins of surgically resected liver. Rat HSCs were isolated from normal livers of Sprague-Dawley rats. Mouse HSC were isolated from normal livers of *UCHL1*$^{-/-}$ or WT littermate controls. Liver tissue was digested with pronase and collagenase B (Roche) and the cell suspension was subsequently separated by an 11.5% Optiprep gradient (Sigma). HSCs were seeded onto plastic (Corning), cultured in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 16% fetal bovine serum, pyruvate, glutamine, penicillin, and streptomycin (Life Technologies) and maintained in an incubator at 37 °C with 5% CO_2. Freshly isolated (day 0) cells were considered quiescent and (day 10) cultures regarded as activated unless specifically stated otherwise.

Histology

Liver tissue sections stained with Haematoxylin-eosin were evaluated by an experienced liver pathologist (DGT) for inflammatory cell infiltrate, severity of necroinflammation including extent and topography of parenchymal necrosis, hepatocyte ballooning and apoptosis, and type and extent of steatosis. A necroinflammatory score (range 0–6) was devised based on the semi-quantification of confluent necrosis by Ishak *et al.* [15]. Staging of fibrosis was assessed according to Ishak *et al.* [15] on sections stained with Sirius red as previously described [16].

Immunohistochemistry

Immunohistochemistry for alpha smooth muscle actin (αSMA) was performed on 4 μm thick formalin-fixed paraffin-embedded sections as previously described [14]. For UCHL1, slides were deparaffinised, rehydrated through graded alcohols, endogenous peroxidase was blocked in hydrogen peroxide/methanol and antigen retrieval was performed using citric saline for 20 min in the microwave, followed by incubation in trypsin for 10 min at 37 °C. Sections were blocked using the Vector avidin/biotin blocking kit and 20% swine serum, prior to overnight incubation at 4 °C with primary antibody UCHL1 (Serotec) at a 1:100 dilution. Next day slides were washed in PBS and incubated with biotinylated rabbit anti-mouse at 1:2000 (Vector labs) for 2 h. After PBS washing, slides were incubated with streptavidin biotin-peroxidase complex (Vector Labs) and incubated at room temperature for 45 min. UCHL1-positive cells were visualized by 3,3-diaminobenzidine tetrahydrochloride (DAB) and counterstained with haematoxylin.

Hydroxyproline assay

The collagen content of liver tissue was measured using the hydroxyproline method, as previously described [17]. Briefly, liver samples were hydrolysed for 18 h at 110 °C in 1 ml of 6 N HCl. Samples were then neutralised in 10 N NaOH before colourisation with Ehrlich's reagent. A standard curve comprised of dilutions of 400 μM hydroxyproline was used for quantification.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Total protein was fractionated by 12% SDS-PAGE and transferred to nitrocellulose membrane. Blots were blocked with TBS/Tween 20 (0.1% T-TBS) containing 5% milk or 5% BSA protein before overnight incubation with primary antibodies. Primary antibodies raised against UCHL1 (#3524 Cell signalling), USP44 (Santa Cruz 79330), αSMA (A5228 Sigma), p27 (Santa cruz 528), pRb (Cell signalling #9301), total Rb (Santa cruz 74562), and GAPDH (ab22555 Abcam) were used at 1:1000 dilution. Membranes were washed in T-TBS and incubated with anti-rabbit (#7074S, Cell Signalling), horseradish peroxidise (HRP)-conjugate antibodies at 1:2000 dilution for 1 h. Blots were washed and antigen detected by ECL (Amersham Biosciences).