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Autotaxin-lysophosphatidic acid receptor signalling regulates hepatitis C virus replication

Michelle J. Farquhar[1,†], Isla S. Humphreys[1,†], Simon A. Rudge[2,†], Garrick K. Wilson[1], Bishnupriya Bhattacharya[1], Maria Ciaccia[2], Ke Hu[1], Qifeng Zhang[2], Laurent Mailly[3], Gary M. Reynolds[4], Margaret Ashcroft[5], Peter Balfe[1], Thomas F. Baumert[3], Stephanie Roessler[6], Michael J.O. Wakelam[2,‡], Jane A. McKeating[1,*,‡]

[1]*Viral Hepatitis Laboratory, Centre for Human Virology, University of Birmingham, UK;* [2]*The Babraham Institute, Cambridge, UK;* [3]*INSERM U1110, University of Strasbourg, 3 Rue Koeberlé, F-67000 Strasbourg, France;* [4]*NIHR Liver Biomedical Research Unit, University of Birmingham, Birmingham, UK;* [5]*Cambridge Biomedical Campus, University of Cambridge, Cambridge, UK;* [6]*Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany*

Background & Aims: Chronic hepatitis C is a global health problem with an estimated 170 million hepatitis C virus (HCV) infected individuals at risk of progressive liver disease and hepatocellular carcinoma (HCC). Autotaxin (ATX, gene name: *ENPP2*) is a phospholipase with diverse roles in the physiological and pathological processes including inflammation and oncogenesis. Clinical studies have reported increased ATX expression in chronic hepatitis C, however, the pathways regulating ATX and its role in the viral life cycle are not well understood.

Methods: *In vitro* hepatocyte and *ex vivo* liver culture systems along with chimeric humanized liver mice and HCC tissue enabled us to assess the interplay between ATX and the HCV life cycle.

Results: HCV infection increased hepatocellular ATX RNA and protein expression. HCV infection stabilizes hypoxia inducible factors (HIFs) and we investigated a role for these transcription factors to regulate ATX. *In vitro* studies show that low oxygen increases hepatocellular ATX expression and transcriptome analysis showed a positive correlation between *ATX* mRNA levels and hypoxia gene score in HCC tumour tissue associated with HCV and other aetiologies. Importantly, inhibiting ATX-lysophosphatidic acid (LPA) signalling reduced HCV replication, demonstrating a positive role for this phospholipase in the viral life cycle. LPA activates phosphoinositide-3-kinase that stabilizes HIF-1α and inhibiting the HIF signalling pathway abrogates the pro-viral activity of LPA.

Conclusions: Our data support a model where HCV infection increases ATX expression which supports viral replication and HCC progression.

Lay summary: Chronic hepatitis C is a global health problem with infected individuals at risk of developing liver disease that can progress to hepatocellular carcinoma. Autotaxin generates the biologically active lipid lysophosphatidic acid that has been reported to play a tumorigenic role in a wide number of cancers. In this study we show that hepatitis C virus infection increases autotaxin expression via hypoxia inducible transcription factor and provides an environment in the liver that promotes fibrosis and liver injury. Importantly, we show a new role for lysophosphatidic acid in positively regulating hepatitis C virus replication.
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Introduction

Chronic viral hepatitis is a global health problem with at least 170 million hepatitis C virus (HCV) infected individuals at risk of developing liver disease that can progress to hepatocellular carcinoma (HCC). The recent availability of direct-acting antiviral agents can eliminate HCV in up to 90% of patients [1]. However, the high cost of these drugs along with reports of viral genotype resistance, may limit their wide-spread use. Importantly, patients with liver cirrhosis cured of HCV may remain at risk of developing HCC, highlighting the need to understand host pathways playing a role in HCC development [2,3].

Autotaxin (ATX. Gene name: *ENPP2*) is a member of the ectonucleotide pyrophosphatase/phosphodiesterase family of proteins that was identified as a motility-stimulating factor secreted from melanoma cells [4]. ATX hydrolyzes lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA), a growth factor that activates and signals via a family of six G-protein coupled LPA receptors (LPA_{1-6}). The ATX-LPA signalling axis has been reported to play a tumorigenic role in a wide number of cancers and is a candidate for therapeutic intervention [5]. Several studies have reported increased ATX and LPA levels in the plasma of HCV infected subjects that associates with liver fibrosis staging [6–9]. A recent prospective study showed that serum ATX

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* Corresponding author. Address: Nuffield Department of Medicine, University of Oxford, Headington, Oxford, UK; Tel.: +44 1865 612 894.
E-mail address: jane.mckeating@ndm.ox.ac.uk (J.A. McKeating).
† These authors have co-first authorship.
‡ These authors have co-senior authorship.

predicts the severity of liver cirrhosis and prognosis of patients with cirrhosis [10]. Mazzocca and colleagues reported that HCC secreted LPA increases the trans-differentiation of peritumoral fibroblasts to carcinoma associated fibroblasts that are considered to play a role in tumour proliferation and metastasis [11].

ATX is expressed in many tissues and the mechanisms accounting for increased serum phospholipase activity in chronic hepatitis C and the impact on viral replication are not understood. We show that HCV infection of hepatocyte-derived cells or mice with humanized chimeric livers increases ATX mRNA and protein expression. Infection stabilizes hypoxia inducible factor-1α (HIF-1α) [12,13] and we show that low oxygen increases ATX transcripts in human liver slices, suggesting a pathway for HCV to regulate ATX. We demonstrate a positive association between *ATX* and hypoxia related gene expression in viral and non-viral HCC, providing an explanation for elevated ATX expression in tumours that are frequently hypoxic. Finally, we demonstrate that ATX-LPA signalling regulates HCV RNA replication via a phosphoinositide 3 kinase (PI3K) dependent pathway, demonstrating a role for lysophospholipids in viral infection. Our data support a model where HCV infection increases hepatocellular ATX expression that promotes viral replication and establishes a paracrine LPA signalling environment leading to fibrosis and HCC pathogenesis.

Materials and methods

Cell lines, antibodies and reagents

HuH7 (provided by Charles Rice, The Rockefeller University, NY, USA) and 293T (American Type Culture Collection) cells were propagated in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% non-essential amino acids (Invitrogen, CA). HuH7 Luc2a-JFH cells (provided by Robert Thimme, Freiburg) [21] were propagated in the same media supplemented with G418. All cells were grown at 37 °C in 5% CO_2 and monitored for mycoplasma contamination using a commercial kit (MycoAlert, Lonza). For hypoxic conditions cells were cultured at 37 °C in a humidified sealed H35 Hypoxystation (Don Wiley Scientific, UK) set to 5% CO_2/95% N_2/1% O_2.

The primary antibodies were: anti-NS5A 9E10 (C. Rice, Rockefeller University, USA); anti-ATX 4FAB; anti-AKT and anti-pAKT (Cell Signaling); anti-HIF-1α (BD Biosciences, Europe). Secondary labelled antibodies: Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, CA); Horseradish peroxidase conjugated sheep anti-mouse and donkey anti-rabbit (GE Healthcare, UK) and anti-rat secondary antibodies (Jackson laboratories). Agonists, inhibitors and antagonists were obtained from the following sources: HA130 (Echelon Biosciences), LPA (Oleoyl-L-alpha-lysophosphatidic acid sodium salt) and wortmannin (Sigma), Ki16425 (Cayman Chemical), BYL-719 (Active Biochem) and TGX-221 (Cayman Chemicals). Cell lysates were quantified for protein content using a standard Bradford assay and 40 µg of protein analysed. For quantitation of secreted ATX, 50 µl of extracellular serum-free conditioned media harvested from a defined cell number was analysed.

Solvents and chemicals for lipids analysis were purchased from the following suppliers: 13:0 LPA (1-tridecanoyl-sn-glycero-3-phosphate in methanol, Avanti Polar lipids-Stratech Scientific Limited), butanol Chromasolv Plus for HPLC and ammonium formate for mass spectrometry (Sigma), acetonitrile and water ultra-gradient grade (Romil), formic acid Optima LC/MS grade (Fisher Scientific).

uPA-SCID mice infection and immunohistochemical ATX staining

uPA/SCID-bg mice were transplanted with primary human hepatocytes (PHH) at 3 weeks of age by intrasplenic injection as described [14]. Engraftment was assessed by measuring human serum albumin and inoculated with HCV J6/JFH (Jc1) virus. Mice were sacrificed at 16 weeks, the liver recovered and frozen for RNA extraction. Liver samples were fixed in formalin for immunostaining purposes. Experiments were performed at Inserm Unit 1110 animal facility according to local laws and approved by the ethical committee of Strasbourg (number AL/02/19/08/12 and AL/01/18/08/12). Sections (3 µm) of formalin fixed paraffin-embedded liver tissue were deparaffinised, rehydrated and after a low temperature retrieval technique and immunostained for ATX using a Dako Autostainer. Bound antibody was detected with rabbit anti-rat secondary for 15 min, ImmPRESS rabbit secondary for 30 min and visualised using ImmPACT DAB (Vector Labs, UK) and counterstained with Meyers haematoxylin.

RT-PCR quantification of ATX and HIF-target genes

Gene amplification was performed in a single tube RT-PCR in accordance with manufacturer's guidelines (CellsDirect kit, Invitrogen, CA) and fluorescence monitored in a 7900HT real time PCR machine (ABI, CA). The housekeeping gene *GAPDH* was included as an internal control for amplification efficiency and RNA quantification (primer-limited endogenous control, ABI).

HCC liver samples, clinical data and gene expression data

We used an Affymetrix U133A2.0 gene expression data set derived from 247 HCC patients as described [16] (GSE14520). Patient samples were obtained with informed consent from patients at the Liver Cancer Institute and Zhongshan Hospital (Fudan University, Shanghai, China). This cohort contained paired tumour and adjacent non-tumour samples from 232 patients. We also performed gene expression analysis on a German cohort from Heidelberg University Hospital [15]. These tissues included tumour tissue of HCC patients with underlying alcoholic liver disease (ALD, N = 8) and hepatitis B virus (HBV) (N = 8) or HCV (N = 9) infection and normal liver samples of patients without HCC or liver cirrhosis (NL, N = 7).

Ex vivo liver slices

Liver tissue was obtained from patients undergoing resection or transplantation surgery at the Queen Elizabeth Hospital, Birmingham. All liver samples were collected with local National Health Service research ethics committee approval (Walsall LREC 04/Q2708/40) and written informed consent. Cores were cut from the tissue immediately upon receipt in the laboratory. A Krumdieck Tissue Slicer (Alabama Research and Development, USA) was used to section the liver cores. Briefly, the core was placed into the slicer under aseptic conditions and circular slices of ~240 µm thickness generated. Slices were immediately transferred into Williams E media (Sigma, UK) supplemented with 1% L-glutamine and 0.5 µM insulin.

ATX promoter activity

Forward (5′CCGGTACCTGTGCTGCGGAAGAAAAGATG3′) and reverse (5′GCCTCGAGGAAAGCCTTTAGCGTG3′) primers were used to amplify the ATX promoter region from HepG2 genomic DNA. PCR fragments were cloned into luciferase reporter plasmid pGL4.28 (Promega, Madison, WI) digested with *Kpn*I and *Xho*I (pGL4.ATX.luc). HuH7 cells were transfected with pGL4.*ATX*.luc or pHRE-Luc and 24 h later re-seeded in 96 well plates and incubated under 20% O_2 or 1% O_2 for 24 h (Don Whitley Scientific Limited). Cells were lysed and luminescence measured.

Mass spectrometric LPA analysis

HuH7 cells were serum starved for 8 h and supernatants harvested, clarified and spiked with 1 ng of 13:0 LPA as an internal standard prior to extraction with 500 µl of butanol. The combined butanol layers were dried under reduced pressure and re-suspended in 100 µl of chloroform/methanol/water 2:5:1 (v/v/v). 5 µl of each sample was analysed using a Shimadzu Prominence HPLC connected to a QTrap equipped with an electrospray ionisation source (AB Sciex 6500). Separation of LPA species from other interfering lipids such as LPS and LPC was achieved using a Cogent Diamond Hydride column (1 x 150 mm, 4 µm, Microsolv) with the following conditions: 0.2 ml/min flow rate, column temperature 40 °C, autosampler temperature 21 °C. Solvent A was 5 mM ammonium formate aqueous solution pH 3.5 and solvent B was acetonitrile containing 0.1% formic acid and 1% of a 200 mM aqueous solution of ammonium formate pH 3.5. Gradient elution was as follows: isocratic 100% B for 4 min, linear decrease 100–75% B in 1.5 min, isocratic 75% B for 3.5 min, sharp step down to 25% B and isocratic 25% B for 5 min (washing step), followed by 10 min of re-equilibration with 100% B. The mass spectrometer was operated in negative ion mode using multiple reaction monitoring to record the following transitions: 367.2 → 153.0 for 13:0 LPA, 381.2 → 153.0 for 14:0 LPA, 409.2 → 153.0 for 16:0 LPA, 437.3 → 153.0 for 18:0 LPA, 435.3 → 153.0 for 18:1 LPA, 433.2 → 153.0 for 18:2 LPA, 457.2 →

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