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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Netrin-1 Protects Hepatocytes Against Cell Death Through Sustained Translation During the Unfolded Protein Response

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SUMMARY

The unfolded protein response (UPR) is a hallmark of numerous liver diseases including cancer. Here, we report that in the liver, netrin-1 protects against UPR-related cell death through UPR-resistant, internal ribosome entry site–driven translation, and the UNC5/death-associated protein kinase pathway.

BACKGROUND & AIMS: Netrin-1, a multifunctional secreted protein, is up-regulated in cancer and inflammation. Netrin-1 blocks apoptosis induced by the prototypical dependence receptors deleted in colorectal carcinoma and uncoordinated phenotype-5. Although the unfolded protein response (UPR) triggers apoptosis on exposure to stress, it first attempts to restore endoplasmic reticulum homeostasis to foster cell survival. Importantly, UPR is implicated in chronic liver conditions including hepatic oncogenesis. Netrin-1's implication in cell survival on UPR in this context is unknown.

METHODS: Isolation of translational complexes, determination of RNA secondary structures by selective 2'-hydroxyl acylation and primer extension/dimethyl sulfate, bicistronic constructs, as well as conventional cell biology and biochemistry approaches were used on in vitro–grown hepatocytic cells, wild-type, and netrin-1 transgenic mice.

RESULTS: HepaRG cells constitute a bona fide model for UPR studies in vitro through adequate activation of the 3 sensors of the UPR (protein kinase RNA–like endoplasmic reticulum kinase (PERK)), inositol requiring enzyme 1α (IRE1α), and activated transcription factor 6 (ATF6). The netrin-1 messenger RNA 5'-end was shown to fold into a complex double pseudoknot and bear E-loop motifs, both of which are representative hallmarks of related internal ribosome entry site regions. Cap-independent translation of netrin 5' untranslated region–driven luciferase was observed on UPR in vitro. Unlike several structurally related oncogenic transcripts (l-myc, c-myc, c-myb), netrin-1 messenger RNA was selected for translation during UPR both in human hepatocytes and in mice livers. Depletion of netrin-1 during UPR induces apoptosis, leading to cell death through an uncoordinated phenotype-5A/C–mediated involvement of protein phosphatase 2A and death-associated protein kinase 1 in vitro and in netrin transgenic mice.

CONCLUSIONS: UPR-resistant, internal ribosome entry site–driven netrin-1 translation leads to the inhibition of uncoordinated phenotype-5/death-associated protein kinase 1–mediated apoptosis in the hepatic context during UPR, a hallmark of chronic liver disease. *(Cell Mol Gastroenterol Hepatol 2016;2:281–301; http://dx.doi.org/10.1016/j.jcmgh.2015.12.011)*

Keywords: Netrin; UPR; Hepatocyte; Translation.

The endoplasmic reticulum (ER) is the place of secretory and membrane protein synthesis.[1] Folding of newly synthesized proteins within the ER lumen is tightly monitored by dedicated quality control machinery.[2] Perturbation of ER homeostasis caused by hypoxia, viral infection, or other stressors can reduce or surpass the ER folding capacity, resulting in a condition termed *ER stress*.[3] Chronic ER stress is observed in several diseases including cancer.[4–6] Likewise, ER stress plays a well-documented role in hepatitis B virus (HBV)- and hepatitis C virus (HCV)-related pathogenesis,[7–9] implicating it as a factor in liver disease and carcinogenesis.[7,10–12] To restore homeostasis in response to ER stress, cells activate the unfolded protein response (UPR), a process involving the sequential

Abbreviations used in this paper: **ATF6, activated transcription factor 6; CMV, cytomegalovirus; DAPK, death-associated protein kinase; DMS, dimethyl sulfate; DR, dependence receptor; DTT, dithiothreitol; eIF2α, Eukaryotic translation initiation factor 2A; ER, endoplasmic reticulum; FLuc, Firefly luciferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IRE1α, inositol requiring enzyme 1α; IRES, internal ribosome entry site; LSL, (Lox-Stop-Lox); mRNA, messenger RNA; NMIA, N-methyl-isatoic anhydride; pBic, Bicistronic plasmid; PBS, phosphate-buffered saline; PERK, protein kinase RNA (PKR)-like endoplasmic reticulum kinase; PP2A, protein phosphatase 2A; PR65β, erine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; RLuc, Renilla lucerifase; siRNA, small interfering RNA; Tu, tunicamycin; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; UNC5, uncoordinated phenotype-5; UPR, unfolded protein response; UTR, untranslated region; VR1, vanilloid receptor 1.**

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activation of 3 ER sensors named protein kinase RNA (PKR)-like endoplasmic reticulum kinase (PERK), activated transcription factor 6 (ATF6), and inositol requiring enzyme 1α (IRE1α).[13] PERK phosphorylates the elongation factor Eukaryotic translation initiation factor 2A (eIF2α) at Ser-51, impeding protein translation. If the UPR fails to restore ER homeostasis, it instead reverts to apoptosis.[13] One of the mediators of UPR-induced apoptosis is the death-associated protein kinase 1 (DAPK1), a key regulator of cell death.[14] DAPK1 is activated by protein phosphatase 2A (PP2A) in a process that requires interactors including calmodulin, and, interestingly, the Unc-5 homolog B (UNC5B),[15,16] suggesting a potential involvement of extracellular or autocrine factors.

UNC5B is one of 4 members of the UNC5-family (UNC5A, UNC5B, UNC5C, and UNC5D), belonging to the so-called dependence receptors (DRs),[17] promoting cell survival as long as they are engaged by their ligands. Once unbound, these receptors trigger apoptosis.[17–19] Netrin-1 is the canonical soluble partner of DRs. It initially was identified as an axonal guidance molecule of the developing central nervous system.[20] In the past decade, several studies have reported that netrin-1 is upregulated in several types of cancer[19,21–24] and cancer-associated inflammatory diseases conferring cells with a selective advantage regarding survival and proliferation.[25–27] Netrin-1 is upregulated in cancers in general and in cancer-associated associated inflammatory diseases. Intriguingly, netrin-1 is increased by 10- to 30-fold upon HBV or HCV infection in an epidermal growth factor receptor–dependent manner in the latter case, and also in cirrhosis irrespective of its etiology (Plissonnier et al, unpublished data). From what is known, UNC5A and C induce apoptosis through the recruitment of neurotrophin receptor-interacting MAGE homolog or the activation of the E2F Transcription Factor 1 transcription factor, respectively.[15,16] As mentioned earlier, UNC5B binds and signals via DAPK1, triggering a signal cascade that has been well described. Briefly, in the presence of netrin-1, the UNC5B receptor interacts with an inactive, phosphorylated form of DAPK1. In the absence of netrin-1, UNC5B adopts an open conformation and recruits Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform (PR65β)/PP2A into an UNC5B/DAPK1 complex followed by caspase-3 activation.[16] Two recent studies have suggested a link between netrin-1 and the UPR.[28,29] Given the already known association of UNC5B and DAPK1 with this process, we sought to investigate the role of netrin-1/UNC5-controlled apoptosis in UPR-associated cell death.

In the liver, common triggers include viral infections, alcoholic liver disease, or genetic conditions, all of which are high risk factors for hepatocellular carcinoma.[7–9,30–32] The UPR is a hallmark of these pathophysiological contexts. Here, we show that during experimentally induced UPR, netrin-1 is efficiently translated through an internal ribosome entry site (IRES) both in vitro and in vivo in mice livers. Modulation of netrin-1 in hepatocytic cells conditions caspase-3 activation and affects cell death via UNC5A- and UNC5C-mediated increase of PP2A activity and implication of DAPK1. Our results indicate that netrin-1 protects hepatocytes against UPR-related cell death through resistance to UPR-related global translational inhibition.

Materials and Methods

Cell Culture

HepaRG cells were cultured as previously described.[33] The human hepatoma cell line Huh7.5 was grown in Dulbecco's modified Eagle medium (Life Technologies, Carlsbad, CA), supplemented with 10% fetal bovine serum (Thermo Scientific, Waltham, MA), 1× penicillin-streptomycin (Life Technologies), and 1× glutamax (Life Technologies). Cells were maintained in a 5 % CO_2 atmosphere at 37°C and harvested at day 3 after plating. Neutralizing netrin-1 antibody 2F5 and the isotypic control H4 were obtained from Netris Pharma (Lyon, France). ER stress was induced by treating cells with tunicamycin (Tu) (Sigma-Aldrich, St. Louis, MO) or dithiothreitol (DTT) (Sigma-Aldrich) as indicated before harvest.

Mice Models

All trials were performed under Institutional Review Board agreement CECCAP_CLB_2014_015. Six-week-old C57BL6 mice (Charles River Laboratory, Saint-Germain-Nuelles, France) were treated intraperitoneally with 1 mg/kg Tu or phosphate-buffered saline (PBS) for 24 hours and killed. Rosa-Lox-Stop-Lox (LSL)-netrin-1 transgenic mice conditionally overexpress flag-tagged netrin-1 under the control of a Rosa26 promoter. These animals were crossed with Rosa-CreERT2 (tamoxifen-dependent Cre recombinase) +/+ mice to generate breeder pairs of control and conditional overexpressers. Each mouse carries one copy of the CreERT2 transgene and was genotyped for LSL–netrin-1. At the age of 8 weeks, mice were injected intraperitoneally with 100 μL of 10 mg/mL tamoxifen (diluted in corn oil/ethanol, 9/1) daily, for 3 consecutive days to induce netrin-1 overexpression. After 2 weeks, mice were genotyped and netrin-1–overexpressing mice and their breeder pairs of control were treated with Tu or PBS for UPR induction for 24 hours and then killed.

Quantitative and Conventional Reverse-Transcription Polymerase Chain Reaction

For quantitative reverse-transcription polymerase chain reaction (qRT-PCR), total RNA was extracted from cultured cells using the Extract-all reagent (Eurobio, Courtaboeuf, France) or the Nucleospin RNA/protein kit (Macherey-Nagel, Duren, Germany) for liver samples. RNA (1 μg) was treated with DNase I (Promega, Madison, WI), and then reverse transcribed in the presence of 5% dimethyl sulfoxide, using the Moloney Murine Leukemia Virus Reverse Transcriptase enzyme, according to the manufacturer's instructions (Invitrogen). Real-time qRT-PCR was performed on a LightCycler 480 device (Roche, Basel, Switzerland) using the iQTM SYBR 533 Green Supermix (Bio-Rad, Hercules, CA). Dimethyl sulfoxide (10%; Sigma-Aldrich) was added to the PCR reaction for human netrin-1 quantification. Conventional RT-PCR was performed to amplify unspliced and spliced forms of XBP1 messenger RNA (mRNA), using the GoTaq DNA Polymerase according to the