Research Article

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A hedgehog survival pathway in 'undead' lipotoxic hepatocytes

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Background & Aims: Ballooned hepatocytes in non-alcoholic steatohepatitis (NASH) generate sonic hedgehog (SHH). This observation is consistent with a cellular phenotype in which the cell death program has been initiated but cannot be executed. Our aim was to determine whether ballooned hepatocytes have potentially disabled the cell death execution machinery, and if so, can their functional biology be modeled *in vitro*.

Methods: Immunohistochemistry was performed on human NASH specimens. *In vitro* studies were performed using HuH-7 cells with shRNA targeted knockdown of caspase 9 (shC9 cells) or primary hepatocytes from *caspase* $3^{-/-}$ mice.

Results: Ballooned hepatocytes in NASH display diminished expression of caspase 9. This phenotype was modeled using shC9 cells; these cells were resistant to lipoapoptosis by palmitate (PA) or lysophosphatidylcholine (LPC) despite lipid droplet formation. During lipid loading by either PA or LPC, shC9 cells activate JNK which induces SHH expression via AP-1. An autocrine hedgehog survival signaling pathway was further delineated in both shC9 and *caspase* $3^{-/-}$ cells during lipotoxic stress.

Conclusions: Ballooned hepatocytes in NASH downregulate caspase 9, a pivotal caspase executing the mitochondrial pathway of apoptosis. Hepatocytes engineered to reduce caspase 9 expression are resistant to lipoapoptosis, in part, due to a hedgehog autocrine survival signaling pathway.

Keywords: Caspase 3; c-Jun-N-terminal kinase (JNK); Lipoapoptosis; Lysophosphatidylcholine; Palmitate.

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Abbreviations: NASH, non-alcoholic steatohepatitis; SHH, sonic hedgehog; FFA, free fatty acids; PA, palmitate; LPC, lysophosphatidylcholine; JNK, c-Jun N-terminal kinase; shRNA, short-hairpin RNA; MPH, mouse primary hepatocytes; D-API, 4′,6-diamidine-2′-phenylindole dihydrochloride; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; OA, oleate; $C3^{-/-}$, caspase 3 genetic knockout; AP-1, activator protein-1; ACOX-1, acyl-CoA oxidase 1, palmitoyl; CPT, carnitine palmitoyltransferase; ACACA, acetyl-CoA carboxylase alpha; ACACB, acetyl-CoA carboxylase beta.

Introduction

Human hepatocellular injury is ubiquitous due to the prevalence of steatohepatitis syndromes, viral hepatitis, immune mediated insults, genetic diseases, etc. [1]. In these hepatic diseases, hepatocyte fate is often modeled as a binary process – cells either survive or die to be replaced by liver regenerative processes [2]. Yet, cells may resist cell death by downregulating or inhibiting cell death programs, thereby surviving in an altered state. This new cell fate does not merely reflect an escape from death, but rather results in the formation of a unique, functional cell phenotype. The cell phenotype of non-lethal cell injury has been best characterized in model systems where it has been termed the 'undead cell' [3,4]. Because cell death by proapoptotic stimuli requires activation of executioner caspases [5], the undead cell can be engineered by deletion of these downstream caspases. For example, in *Drosophila melanogaster* genetic deletion of the executioner caspase DrICE or Dcp-1 results in a cell type in which otherwise toxic stimuli activate alternative cell signaling cascades producing signals which enhance tissue remodeling [3,4,6].

A candidate undead cell in the liver is the ballooned hepatocyte in non-alcoholic steatohepatitis (NASH). Ballooned hepatocytes are thought to be important in the pathogenesis of NASH and the presence of such cells is used to score disease severity [7,8]. Little is known about ballooned hepatocytes other than they are enlarged cells which have lost cell polarity, frequently contain Mallory–Denk bodies, store neutral triglycerides, contain oxidized phospholipids, no longer stain for cytokeratin 18, and generate the ligand sonic hedgehog [8–10]. The seminal work by Diehl and colleagues demonstrating sonic hedgehog (SHH) generation by ballooned hepatocytes suggests this cell may, in fact, be a functional cell with an altered phenotype [8,11]. For example, in *D. melanogaster* retinal cells in which the cell death program is initiated but cannot be executed also generate SHH [6]. The mechanisms by which undead cells continue to evade cell death remain elusive, but given the potency of SHH as a survival factor, it is possible that this ligand serves as an autocrine survival factor.

Herein, we demonstrate that ballooned cells have reduced expression of a potent downstream caspase, caspase 9, suggesting they may not efficiently execute cell death pathways. To understand the functional implications of this observation, we modeled lipotoxic stress in HuH-7 cells in which caspase 9 had been knocked down by short hairpin RNA technology (shC9 cells). Knockdown of caspase 9 protected hepatocytes from lipotoxicity by the saturated free fatty acid (FFA), palmitate (PA) or the

phospholipid lysophosphatidylcholine (LPC). Interestingly, PA or LPC treated shC9, but not wild type cells, generate sonic hedgehog by a c-Jun-N-terminal kinase (JNK)-dependent pathway. Blockade of hedgehog signaling resulted in cell death of shC9 cells following exposure to lipotoxic agents. Similar observations were confirmed in primary *caspase* $3^{-/-}$ mouse hepatocytes. We speculate that these engineered cells model the functional phenotype of ballooned hepatocytes, albeit not the specific morphology, and, in turn, support the concept of ballooned hepatocytes as an altered, but functional cell population which has escaped cell death.

Materials and methods

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded liver sections (5 μm thick) from three patients with non-alcoholic steatohepatitis (NASH) were obtained following approval by the Institutional Review Board (IRB), Mayo Clinic, Rochester MN, USA. In conjunction with the clinical core of P30DK084576, the sections were pre-selected by an experienced hepatopathologist for their abundance of ballooned hepatocytes. These sections were deparaffinized in xylene and rehydrated through graded alcohols. After tissue permeabilization in 0.1% Triton X100 for 2 min, antigen retrieval was performed by incubation in sodium citrate buffer (0.01 M sodium citrate, 0.05% Tween 20; pH 6.0) for 30 min utilizing a vegetable steamer. Samples were cooled down, and endogenous peroxidase activity was quenched by incubation for 15 min in 3% H_2O_2 (diluted in deionized H_2O). Sections were washed in changes of phosphate-buffered saline (PBS), three times for 5 min. The VECTASTAIN Elite ABC and ImmPACT VIP peroxidase substrate kits (Vector laboratories, Burlingame, CA, USA) were used in further steps according to manufacturer's instructions. After blocking non-specific binding for 1 h, sections were incubated with anti-caspase-9 p10 (F-7) mouse monoclonal primary antibodies (1:50; sc-17784) or anti-caspase-2_L (C-20) goat monoclonal primary antibodies (1:50; sc-626-G, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at room temperature. Prior to mounting, the sections were counterstained with hematoxylin for 3 min (Sigma–Aldrich, St. Louis, MO, USA). Samples were examined with light microscopy. Analysis of at least 20 cells of each type from each patient was conducted using Image J version 1.44 software (NIH, Bethesda, MD, USA). Ballooned cells were confirmed in hematoxylin and eosin (H&E) stained sections.

Cells

HuH-7 cells, a human hepatocellular carcinoma cell line, were maintained in Dulbecco's modified Eagle's medium containing glucose (25 mM) supplemented with 10% fetal bovine serum, 100,000 IU/L penicillin and 100 mg/L streptomycin. We also employed HuH-7 cells stably expressing a short-hairpin RNA (shRNA) targeting caspase 9, shC9 cells. Mouse primary hepatocytes (MPH) were isolated from C57BL/6 wild type (Jackson Laboratory, Bar Harbor, ME, USA) and *caspase* $3^{-/-}$ mice by collagenase perfusion and plated as primary cultures [12]. Development and characterization of the *caspase* $3^{-/-}$ mice has been previously reported [13]. MPH were maintained in Waymouth medium supplemented with 10% fetal bovine serum, 100,000 IU/L penicillin, 100 mg/L streptomycin, and 100 nM insulin.

Phase contrast, fluorescent and confocal microscopy

HuH-7 cells and shC9 cells were grown on glass bottom culture dishes for phase contrast and fluorescence microscopy, or glass coverslips for confocal microscopy. The following studies were conducted in conjunction with the microscopy core of the P30DK084567. For live cell imaging, lipid droplets were fluorescently labeled by incubation with 100 μg/ml of Bodipy 505/515 (Invitrogen, Camarillo, CA, USA) for 1 min at 37 °C. Identical cells were viewed using phase contrast and then fluorescent optics on a Zeiss Axiovert 200 epifluorescent microscope (Zeiss, Thornwood, NJ, USA). Fluorescence was visualized using excitation and emission wavelengths of 488 and 507 nm, respectively. Both phase contrast and fluorescent digital images were acquired and analyzed using a cooled-CCD Orca II camera (Hamamatsu Photonics, Hamamatsu City, Japan) with IPLab software (Scanalytics, Fairfax, VA, USA). Fluorescent lipid droplet area as a percent of the total cellular area was quantified using Image J version 1.44 software (NIH, Bethesda, MD, USA).

In separate studies, cells were fixed with 3% paraformaldehyde for 30 min at room temperature [14]. Intracellular lipid droplets were labeled with 10 μg/ml of Bodipy 505/515 for 10 min at room temperature, and mounted in Prolong Antifade mounting media (Invitrogen, Camarillo, CA, USA). Lipid droplet fluorescence intensity per cell was quantified using an inverted phase/fluorescence microscope (Axiovert 35M; Carl Zeiss, Inc., Thornwood, NY, USA) and Metafluor quantitative fluorescence imaging software from Universal Imaging Corp. (West Chester, PA, USA) [15]. For confocal microscopy, cells were fixed and labeled as described above and visualized using an inverted laser-scanning confocal microscope (Zeiss LSM 510, Carl Zeiss, Jena, Germany) with excitation and emission wavelengths of 488 and 507 nm, respectively.

For SHH or ubiquitin immunocytochemistry, cells plated on coverslips were fixed with 4% paraformaldehyde in PBS for SHH staining or with cold acetone on dry ice for ubiquitin staining, respectively, and permeabilized with 0.0125% (w/v) CHAPS in PBS for both stainings. The primary antiserum was rabbit anti-SHH (sc-9024, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:300 or rabbit anti-ubiquitin (Z0458, Dako, Carpinteria, CA, USA) at a dilution of 1:500. The secondary antiserum was Alexa Fluor 488-conjugated or 568-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR, USA), and ProLong Antifade with DAPI (Molecular Probes) was used as mounting medium. Images were acquired by confocal microscopy employing excitation and emission wavelengths of 488 and 507 nm for SHH staining or excitation and emission wavelengths of 558 and 583 nm for ubiquitin staining. Cells with positive immunoreactivity for the active conformation of SHH or cells with ubiquitin-punctate staining, respectively, were counted and expressed as a percentage of total cells.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described by us in detail [16]. Briefly, nuclear extracts from shC9 cells were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. For the EMSA, 20 μg of nuclear proteins was incubated at room temperature for 20 min in binding buffer (25 mM HEPES, pH 7.5, 0.1 M NaCl, 2 mM EDTA, 6% glycerol, 0.1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 0.4 mM dithiothreitol, 0.5 μg/μl poly(dI-dC), 0.5 μg/μl salmon sperm) with 0.04 pmol of CY 5.5-labeled double-stranded DNA oligonucleotides containing either one of the putative activator protein-1 (AP-1) binding sequences (BS1) or (BS2). Each sequence with the mutated putative AP-1 binding sequence (mutant BS1 or mutant BS2) was also employed as a negative control (Supplementary Table 1). Protein-DNA complexes were separated from the unbound DNA probe by electrophoresis through 5% native polyacrylamide gels containing 0.5x Tris borate-EDTA. Fluorescence was visualized directly on the gel using an Odyssey fluorescent imager (Licor Biosciences, Lincoln, NE, USA). For competition assays, a 200-fold molar excess of unlabeled double-stranded oligonucleotide was added to the reaction mixture 20 min before the addition of the fluorescent probe. In the supershift assays, nuclear cell extract was first incubated at room temperature for 25 min with 2 μg of anti-phospho-c-Jun antibody (Santa Cruz Biotechnology). The entire antibody/protein mixture was then incubated with CY 5.5-labeled probe and processed for the gel shift as described above.

Chromatin immunoprecipitaion (ChIP) assay

ChIP assays were performed employing shC9 (6–10 million cells) using a commercially available assay (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions [17]. Briefly, shC9 cells were treated for 6 h with 800 μM palmitate or for 4 h with 85 μM LPC; the cells were then fixed with 1% formaldehyde in PBS at room temperature for 10 min. The formaldehyde crosslinking reaction was quenched with 125 mM glycine, the cells were lysed, and chromatin was sheared into small and uniform fragments by an enzymatic reaction. Following centrifugation, the DNA concentration in the supernatant was quantified by measuring absorbance at 260 nm. Samples containing 30 μg of DNA were next incubated with Protein G magnetic beads, ChIP Buffer 1, Protease Inhibitor Cocktail, the phospho-c-Jun antibody at 2 μg and dH_2O at 4 °C overnight. The immune complex was separated from the sample buffer by a magnetic stand and washed sequentially with ChIP Buffer1 and ChIP Buffer 2. The sample was next eluted with Elution Buffer AM2 and then added to the Reverse Cross-linking Buffer and heated at 95 °C for 15 min. The proteins were digested by proteinase K, and the DNA in the ChIP sample was used as template for quantitative real-time PCR. The primers used in this assay are depicted in Supplementary Table 2.