Research Article

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Perilipin discerns chronic from acute hepatocellular steatosis

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Background & Aims: Hepatocellular steatosis is the most frequent liver disease in the western world and may develop further to steatohepatitis, liver cirrhosis and hepatocellular carcinoma. We have previously shown that lipid droplet (LD)-associated proteins of the perilipin/PAT-family are differentially expressed in hepatocyte steatosis and that perilipin is expressed *de novo*. The aim of this study was to determine the conditions for the temporal regulation of *de novo* synthesis of perilipin *in vitro* and *in vivo*.

Methods: Immunohistochemical PAT-analysis was performed with over 120 liver biopsies of different etiology and duration of steatosis. Steatosis was induced in cultured hepatocytic cells with combinations of lipids, steatogenic substances and DMSO for up to 40 days under conditions of stable down-regulation of adipophilin and/or TIP47.

Results: Whereas perilipin and adipophilin were expressed in human chronic liver disease irrespective of the underlying etiology, in acute/microvesicular steatosis TIP47, and MLDP were recruited from the cytoplasm to LDs, adipophilin was strongly increased, but perilipin was virtually absent. In long-term steatosis models *in vitro*, TIP47, MLDP, adipophilin, and finally perilipin were gradually induced. Perilipin and associated formation of LDs were intricately regulated on the transcriptional (PPARs, C/EBPs, SREBP), post-transcriptional, and post-translational level (TAG-amount, LD-fusion, phosphorylation-dependent lipolysis). In long-term steatosis models under stable down-regulation of adipophilin and/or TIP47, MLDP substituted for TIP47, and perilipin for adipophilin.

Conclusions: LD-maturation in hepatocytes *in vivo* and *in vitro* involves sequential expression of TIP47, MLDP, adipophilin and finally perilipin. Thus, perilipin might be used for the differential diagnosis of chronic *vs.* acute steatosis.

Keywords: PAT-protein family; Lipid droplet; Steatohepatitis; Adipophilin; MLDP; TIP47.

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Abbreviations: ADRP, adipose differentiation-related protein; AFLD, alcoholic fatty liver disease; ASH, alcoholic steatohepatitis; CE, cholesterol-ester; C/EBP, CCAAT/enhancer-binding protein; DMSO, dimethylsulfoxide; HFD, high fat diet; HSL, hormone-sensitive lipase; IRI, ischemia-reperfusion injury; LD, lipid droplet; LTX, liver transplantation; MLDP, myocardial lipid droplet protein; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PAT, perilipin/adipophilin/TIP47-protein-family; PPAR, peroxisome proliferator activating-receptor; RT, room temperature; TAG, triacylglyceride; TIP47, tail-interacting protein 47 kDa.

Introduction

Hepatocellular steatosis is the most frequent liver pathology in western countries and the consequence of various nutritional factors and associated diseases such as alcoholic fatty liver disease (AFLD), obesity, and type II diabetes (non-alcoholic fatty liver disease/NAFLD), chronic hepatitis C (HCV), genetic disorders like lipodystrophy or Wilson's disease, as well as medication with, e.g., tamoxifen or corticosteroids, but also hypoxic injury, parenteral nutrition, and starvation. AFLD and NAFLD may progress to steatohepatitis (ASH, NASH), liver cirrhosis and even hepatocellular carcinoma (HCC) [1]. Histologically, hepatocellular steatosis is defined by the accumulation of lipid droplets (LDs) in hepatocytes concomitant with an increase of triacylglyceride (TAG) content to more than 5% of liver weight.

Intracellular LDs generally consist of a TAG- and/or cholesterol ester-(CE) rich core, which is surrounded by a phospholipid monolayer and associated amphiphilic proteins conferring a LD-specific protein composition dependent on the origin and metabolic status of a cell. LDs are metabolically active, highly dynamic organelles as they represent the regulatory site for neutral lipid hydrolysis and storage [2]. The perilipin/PAT-protein family (concerning nomenclature see [3]), including perilipin (perilipin1) [4], adipophilin (perilipin2, ADRP) [5], TIP47 (perilipin3) [6], S3–12 (perilipin4) [7], and MLDP (perilipin5, OXPAT [3,8]) play the most important role in the biogenesis, stabilization, and degradation of LDs. The exchangeable PAT-proteins (ePATs) TIP47 and MLDP are stable in the cytosol and recruited to LDs under certain metabolic conditions. In contrast, perilipin and adipophilin, the two constitutively expressed PATs (cPATs), only exist bound to LDs; otherwise they are prone to degradation. Adipophilin and TIP47 are almost ubiquitously expressed, whereas perilipin is specific for adipocytes, sebaceous gland epithelial cells, steroidogenic cells, steatotic hepatocytes, and derived tumors [9]. In adipocytes, perilipin promotes lipolysis via hormone-sensitive lipase that is

regulated by catecholamines and protein kinase A in a phosphorylation-specific manner [10]. Perilipin-deficient mice are resistant to obesity [11,12], mice with absence of adipophilin show a hepatic TAG-reduction by more than 50% and a higher metabolic rate compared to control mice [13,14]. Recently, two heterozygous *PLIN1*-frameshift mutations (loss-of-function) were described in patients with partial lipodystrophy, severe dyslipidemia, insulin-resistant diabetes, and hepatic steatosis [15].

We have previously shown that the PAT-proteins perilipin, adipophilin, and TIP47 are differentially expressed in hepatocyte steatogenesis and tumorigenesis *in situ* [16,17]. Perilipin was unexpectedly negative in some, though moderately steatotic human liver specimens, in cultured cells [18], and fatty livers of morbidly obese mice [19], but found positive in a mouse model of chronic, but not acute ethanol ingestion [20]. Therefore, the circumstances of perilipin induction remain unclear. Thus, the aim of this study was to determine the conditions for the temporal regulation of *de novo* synthesis of perilipin both in cell culture and *in vivo*.

Materials and methods

Tissues and cultured cells

Cryopreserved and formalin-fixed, paraffin-embedded human liver specimens were provided by the tissue bank of the National Center for Tumor Diseases (NCT, Heidelberg, Germany; Supplementary Table 1). Written informed consent was obtained from each patient in accordance with the regulations of the tissue bank and the approval of the ethics committee of the University of Heidelberg (No. 206/2005). Human cell lines were cultured and seeded as listed in Supplementary Table 2. Substances and their working concentrations are listed in Supplementary Table 3.

Antibodies and reagents

Primary and secondary antibodies as well as fluorescent dyes are listed in Supplementary Table 4.

Immunofluorescence microscopy and immunohistochemistry

Immunofluorescence microscopy of cultured cells and frozen tissues, and immunohistochemistry of formalin-fixed, paraffin-embedded tissue was performed as published [16,17]. Epifluorescence was done with an Olympus IX81 photomicroscope (Hamburg, Germany). Confocal laser scanning microscopy was performed with a Nikon AR1 microscope (Duesseldorf, Germany).

Gel electrophoresis and immunoblotting

Cultured cells were harvested in cell lysis buffer (Cell Signaling/New England Biolabs, Frankfurt, Germany), and equivalent aliquots of whole protein lysates were determined by Bradford assay. SDS-PAGE, protein transfer onto polyvinylidene difluoride membranes and immunoblot analyses were undertaken as described [16,17]. Densitometric evaluation of 3 independent experiments was performed by normalization to actin as loading control and to starting value.

Total RNA isolation, cDNA synthesis, and real-time PCR

Total cellular RNA was extracted from cultured cells using the NucleoSpin RNA II kit according to the manufacturers' protocol (Macherey-Nagel, Düren, Germany). For semiquantitative real-time PCR analysis, 1 μg total RNA was reversely transcribed and cDNA was amplified using Poly-dT primers and Multi Cycler PTC (Biozym, Oldendorf, Germany). A cycling program using TaqMan SYBR® Green-Master Mix (ABImed, Langenfeld, Germany) was applied for real-time PCR analysis (ABI Prism7300, Applied Biosystems, Darmstadt, Germany): 2 min 50 °C, 15 min 95 °C, 40 times repeat 15 s 95 °C and 1 min 60 °C. Triplicate reactions were performed. *Beta-2-microglobulin* was used as endogenous calibrator (for primer sequences see Supplementary Table 5).

DNA isolation, bisulfite conversion, and pyrosequencing

DNA was isolated using Gentra Puregene Kit (Qiagen, Hilden, Germany). 400 ng DNA per sample were sodium bisulfite-converted using EpiTect Bisulfite Conversion Kit (Qiagen). Bisulfite pyrosequencing was performed on PyroMark Q24 (Qiagen) according to standard protocols, templates were amplified using PyroMark PCR Kit (Qiagen) and primer pairs designed with PyroMark Assay Design SW 2.0 (Qiagen; for sequences see Supplementary Table 5) and data evaluated with Pyro Q-CpG 1.0.9 (Biotage).

siRNA- and lentiviral transfection of HuH7 cells

siRNA-treatment was performed as described [18]. For lentiviral transfection, pAPM vector (originated from pALPS) carrying a microRNA-based shRNA cassettes targeting *adipophilin*, *TIP47*, or non-targeting shRNAs were used (see Supplementary Table 5). For double knockdown, the puromycin resistance gene was replaced by a hygromycin B resistance gene from pAHM NT in the *Xba*I/*Not*I restriction enzyme sites. For transfection, 62 μl of 2 M $CaCl_2$ and 500 μl 2× HBS solution was added to 4 ml fresh DMEM; 6.4 μg pAPM, 6.4 μg psPAX.2, and 2.1 μg pMD2.G (3:3:1 ratio) was diluted in DMEM, and added to HEK-293T cells (1.2×10^6 cells/6-cm dish). After 1 d, medium was changed and 4×10^4 HuH7 target cells were seeded into 6-well plates for 1 d. Then, the supernatant of HEK-293T cells was harvested, and 1 ml of filtrated viral supernatant was added to HuH7 cells for 6 h, then replaced with viral supernatant in DMEM for 1 d (1:1 ratio). After repetition of the procedure the following day, fresh DMEM with 20% FBS was applied for 1 d. Selection was carried out using 1.2 μg puromycin/ml DMEM or 400 μM hygromycin B for 10 or 20 days, respectively. After selection, 0.3 μg of puromycin and 100 μM hygromycin B were constantly applied on selected cells.

Results

Perilipin is expressed in chronic steatosis irrespective of the cause

Previously, we had noted that PAT-protein-expression correlated with the degree of steatosis but no obvious differences in PAT-expression were observed between etiologies such as AFLD/ASH and NAFLD/NASH, and chronic hepatitis C [16], yet, some cases of marked microvesicular steatosis showed strikingly low amounts or even total absence of perilipin. Therefore, we extended the immunohistochemical PAT-analysis to other known causes of hepatocellular steatosis. In about 30 different liver specimens, perilipin was detected in steatohepatitis (NASH), and in chronic hepatitis C as expected, as well as in long-standing drug-induced steatosis, e.g., after cortisone- or tetracyclin-therapy, Wilson's disease, and even genetic diseases in newborn children such as in glycogenosis and mitochondriopathy, but not in normal livers (Fig. 1, Table 1A). Thereby, perilipin was detected in livers with chronic steatosis irrespective of etiology, gender, and age.

Perilipin is absent in acute-onset steatosis

Interestingly, we noted that in ballooned cells in NASH (Fig. 1) and in liver specimens with acute injury, like in liver infarcts, ischemia-reperfusion injury (IRI), HCV reinfection, or parenteral nutrition (PN) in liver transplants, as well as in acute drug-induced steatosis, adipophilin was markedly induced and the ePATs TIP47 and MLDP were often recruited from the cytoplasm to small LDs, whereas perilipin was reduced or absent. To analyse time-dependent PAT-expression in human samples with especially acute steatosis, we chose liver biopsies of patients who