Research Article

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Deficiency of insulin-like growth factor 1 attenuates aging-induced changes in hepatic function: Role of autophagy

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Background & Aims: Circulating insulin-like growth factor-1 (IGF-1) plays a pivotal role in mediating the aging process. This study was designed to evaluate the effect of liver IGF-1 deficiency (LID) on aging-induced changes in hepatic function and underlying mechanisms, with a focus on autophagy.

Methods: Plasma and liver samples were obtained from young (3-mo) and aged (24-mo) wild type (WT) and LID mice. Levels of AST, ALT, triglyceride, hepatic lipofuscin, steatosis, fibrosis, and nuclear morphology were analyzed. Western blot was employed to evaluate autophagy. Human HepG2 cells were treated with free fatty acid (FFA) to mimic hepatic aging in the absence or presence of *IGF-1* siRNA. SA-β-gal activity was detected using flow cytometry and a fluorescence microplate reader. GFP-LC3 was used to assess autophagy activity in HepG2 cells.

Results: Median survival was longer in LID mice compared with WT mice. Aging was associated with elevated levels of triglyceride, AST and ALT, lipofuscin accumulation, steatosis, fibrosis and nuclear injury, which were significantly attenuated by liver IGF-1 deficiency. Levels of autophagy were suppressed in senescent livers, the effect was reversed in the liver of IGF-1 deficient mice. In HepG2 cells, FFA induced the accumulation of β-gal, which was dramatically suppressed by IGF-1 knockdown. Importantly, inhibiting autophagy using 3-methyladenine mitigated IGF-1 knockdown-induced preservation of autophagic vacuole formation and inhibition of β-gal accumulation in the presence of FFA in HepG2 cells.

Conclusions: Our data revealed that IGF-1 deficiency ameliorated aging-induced hepatic injury, possibly through preventing a concomitant diminution in autophagy. These data provide new insight into the role of IGF-1 and autophagy in the management of aging-induced hepatic injury.

Introduction

The aging liver is usually associated with morphological and epigenetic changes that lead to hepatic injury, mainly manifested by lipid accumulation, steatosis, fibrosis, and cancer [1,2]. In addition, aging is known to induce nuclear hypertrophy and DNA impairment in hepatocytes [3], which in turn prompt aging-associated chromatin remodeling [4]. Although the precise etiology of hepatic aging remains elusive, a number of studies have indicated that hepatic insulin-like growth factor I (IGF-1), produced in response to growth hormone (GH), may play a pivotal role in regulating lifespan and aging-associated organ deterioration [5,6]. GH is heavily involved in the regulation of the aging process, and hence lifespan [7,8]. Excessive GH has been shown to reduce lifespan, while GH deficiency may extend lifespan [9]. A number of scenarios have been postulated for GH shortage-mediated extension of lifespan, including a reduction in IGF-1 [9]. Ample studies have further confirmed that a decrease in insulin/IGF-1 signaling extends lifespan in various species including *Caenorhabditis elegans*, *Drosophila melanogaster*, and mammals [10–14]. In particular, female IGF-1 receptor gene knockout ($Igfr^{+/-}$) mice exhibited a 33% increase in lifespan [15]. Moreover, a study from our own group has shown that liver IGF-1 deficiency (LID) preserves cardiac function in aging [16]. Along the same line, deficiency in liver-derived IGF-1 was recently reported to increase lifespan in mice [6].

The autophagy-lysosome pathway plays an essential role in protein and organelle degradation and recycling, and is known to perform "house-keeping" actions under physiological and pathophysiological conditions [17]. Through degradation of defective proteins and organelles, autophagy exerts a wide array of cytoprotective actions [18,19]. Defects in the collective processes involved in autophagy contribute to the onset and development of human diseases [19,20]. Accumulating evidence

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Abbreviations: IGF-1, insulin-like growth factor-1; LID, liver IGF-1 deficiency; WT, wild type; FFA, free fatty acid; 3-MA, 3-methyladenine; GH, growth hormone; mTOR, mammalian target of rapamycin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PA, palmitic acid; Baf A1, bafilomycin A1; C_{12}FDG, 5-dodecanoylaminofluorescein Di-β-D-galactopyranoside; CDK, cyclin-dependent kinase; LI, liver.

suggests a rather important role of autophagy in aging and aging-associated organ injuries [21–23]. Whereas defective autophagy has been tied to shortened lifespan, induction of autophagy has been shown to possess anti-aging effects and ameliorate aging-associated anomalies [21,22]. In the senescent liver, autophagy activity was dramatically decreased. Restoration of autophagy has been demonstrated to be protective for the liver [24].

Since aging is commonly associated with altered circulating levels of IGF-1, a key regulator of insulin signaling and autophagy [25], the present study was designed to examine the effect of IGF-1 deficiency on hepatic aging and the underlying mechanism(s) of action involved, with a focus on autophagy. Hepatic injury was evaluated using plasma levels of triglycerides, aspartate aminotransferase (AST), and alanine aminotransferase (ALT). IGF-1 may suppress autophagy in fibroblasts accompanied by mitochondrial injury and reduced long-term viability [25]. By contrast, IGF-1 deficiency promotes autophagy and mitochondrial biogenesis [25]. Therefore, levels of autophagy and autophagy signaling regulator mammalian target of rapamycin (mTOR), the primary target of IGF-1 downstream of Akt [26], were monitored in livers from young or old mice.

Materials and methods

Experimental animals

All animal procedures used in this study were approved by the Animal Care and Use Committee at the University of Wyoming (Laramie, WY) and were in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). Briefly, liver IGF-1 deficient (LID) mice on a mixed C57BL/6, FVB/N, and 129sv background were generated using the Cre/loxP system as described previously [16,27,28]. Genotyping of offspring was performed using a double PCR strategy. Adult mice positive for *IGF-1*/*loxP* and *Cre* transgenes were used as LID and C57BL/6 mice served as the wild type (WT) control [16,27,28] at young (3 mo) and old (24 mo) ages. All mice used for lifespan analysis (log-rank test) were assigned to a longevity cohort at birth and were not used for any other tests. Male mice were used for this study. Plasma concentrations of triglycerides, AST and ALT were measured using commercially available kits (BioVision Inc, Milpitas, CA).

Human HepG2 hepatocytes

Human HepG2 hepatocyte cells were kindly provided by Dr. Mengwei Zang from Boston University School of Medicine (Boston, MA). HepG2 hepatocytes were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY) containing 10% fetal bovine serum (FBS) and 1% antibiotic (streptomycin 100 U/ml and penicillin 100 U/ml, GIBCO), as previously described [29]. HepG2 hepatocyte cells were grown to confluence on a Lab-Tec chamber slide and maintained in the culture media in humidified air with 5% CO_2 at 37 °C.

Drug treatment and in vitro cell senescence model

Palmitic acid, sodium oleate (O7501), and 3-methyladenine (3-MA) were purchased from Sigma (St. Louis, MO). Bafilomycin A1 (Baf A1) was obtained from EMD4Biosciences (Gibbstown, NJ). 5-Dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG), a fluorogenic substrate for β-galactosidase, was purchased from Molecular Probes (Grand Island, NY). Free fatty acid was a mixture of oleate and palmitate (2:1). 3-MA is a class III phosphatidylinositol 3-kinase inhibitor that inhibits autophagy [30]. Bafilomycin A1 (Baf A1) was employed in HepG2 hepatocyte cells for lysosomal alkalinization prior to detection of the SA-β-gal [31]. To induce *in vitro* hepatocyte senescence, human HepG2 hepatocytes were treated with 1 mM FFA for 48 h, followed by standard (DMEM without FFA) culture media in humidified air with 5% CO_2 at 37 °C for an additional 7 days. 3-MA (2.5 mM) was added after cells were released from FFA treatment [32]. To examine autophagic flux, Baf A1 (100 nM) was added to the culture media for 3 h before HepG2 cells were harvested for further analysis [30].

Detection of SA-β-gal activity

Seven days after HepG2 hepatocytes were released from FFA challenge, levels of SA-β-gal were measured as described [31,33]. Lysosomal alkalinization of HepG2 hepatocytes was induced by incubation of Baf A1 (100 nM) in humidified air with 5% CO_2 at 37 °C for 1 h. C_{12}FDG was added in the culture media containing Baf A1 for another 2 h. After fixation with 4% formaldehyde at room temperature, nuclei were stained with DAPI. Following the mounting of coverslips, cells were visualized with an Olympus BX-51 microscope (Olympus America Inc., Melville, NY). To quantify the HepG2 hepatocytes positive for C_{12}FDG, cells were rinsed with PBS, trypsinized, collected, and resuspended in ice cold PBS. The HepG2 hepatocyte suspension was analyzed using a flow cytometer (Millipore Guava easyCyte 8HT, Millipore Corporation, Billerica, MA). The fluorescence intensity was quantified at 485 nm for excitation and 530 nm for emission using a fluorescence microplate reader (SpectraMax Gemini XS, Molecular Devices, San Diego, CA).

mRNA interference of IGF-1 and Atg7

For *IGF-1* or *Atg7* mRNA silencing, human HepG2 hepatocytes were cultured in an antibiotic-free DMEM medium supplemented with 10% FBS. Following overnight incubation, cells were transfected with a human IGF-1-specific small interference RNA (siRNA) or *Atg7*-specific siRNA commercial agents (Thermo SCIENTIFIC Dharmacon RNAi Technologies, Lafayette, CO). This On-TARGET plus SMART pool of *IGF-1* or *Atg7* siRNA is a mixture of four independent siRNAs. Control cells were treated with non-targeting scrambled control siRNA. In all cases, cells were transfected with 50 nM annealed siRNA oligonucleotides using Oligofectamine reagent (Invitrogen) following the manufacturer's protocol. Following incubation with the *IGF-1* or *Atg7* siRNA agent for 48 h at 37 °C, HepG2 hepatocytes were treated with different drugs for further analysis.

Transfection of human HepG2 hepatocytes with GFP-LC3 adenovirus

Human HepG2 hepatocytes were infected with adenoviruses (2 MOI) expressing GFP-LC3 fusion protein [34]. After transfection, HepG2 hepatocytes were treated with or without FFA in the absence or presence of *IGF-1* siRNA and/or 3-MA (2.5 mM). The HepG2 hepatocytes were then fixed with 4% paraformaldehyde for 20 min at room temperature. To evaluate autophagic activity, HepG2 hepatocytes were visualized using a fluorescence microscope. Cells with more than 10 GFP-LC3 puncta were counted [34].

Lipofuscin autofluorescence

Serial sections of liver tissues were cut at 7-μm thickness by a Leica, cryomicrotome (Model CM3050S, Leica Microsystems, Buffalo Grove, IL). Following three washes in phosphate-buffered saline (PBS), the sections were mounted with aqueous mounting media and coverslipped. To measure the level of lipofuscin in the *in vitro* induced senescence model, HepG2 cells were fixed with 4% formaldehyde and nuclei stained with DAPI. For visualization of autofluorescent lipofuscin deposits, unstained liver sections and HepG2 hepatocyte cells were observed using an Olympus BX-51 microscope (Olympus America Inc., Melville, NY) through the FITC light channel.

Lipofuscin assay

Frozen liver tissue was homogenized in chloroform-methanol (1:20, w:v). The chloroform-rich layer was mixed with methanol following 15 min centrifugation at 15,000*g*. The fluorescence in the sample was measured at an excitation wavelength of 350 nm and emission wavelength of 485 nm using a spectrofluorimeter (Molecular Devices) [23,35]. The data was expressed as fluorescence intensity per 100 mg tissue.

Oil Red O staining

The frozen liver was sectioned at 7-μm thickness with a Leica cryomicrotome (Leica Microsystems) prior to fixation in 10% formalin for 10 min. Following three washes with distilled water, the slides were placed in absolute propylene glycol