Downregulation of citrin, a mitochondrial aspartate glutamate carrier, is associated with apoptosis of hepatocytes

Downregulation of citrin, a mitochondrial AGC, is associated with apoptosis of hepatocytes

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Abstract

Citrin is a mitochondrial aspartate glutamate carrier primarily expressed in liver. Adult-onset type II citrullinemia is caused by mutations in the SLC25A13 gene that encodes for citrin, and patients with this condition do not express citrin. We found apoptotic hepatocytes in one such patient. This finding prompted us to investigate the role of citrin in hepatocyte survival. Knockdown of citrin by a vector-based short-hairpin RNA technique reduced cell viability and induced apoptosis of a hepatocellular carcinoma cell line, Hep3B cells. Caspase-3/7 and caspase-9 were activated, and PARP was cleaved. Citrin knockdown also increased the expression of Bax and Bak, and reduced expression of Bcl-xL and Bcl-2. These alterations resulted in the release of cytochrome *c* from the mitochondria. Our results indicated that citrin downregulation induces apoptosis of hepatocytes through the mitochondrial death pathway, highlighting the importance of citrin in survival of hepatocytes and maintenance of liver function.

Keywords: Apoptosis; Caspase; Citrullinemia; Mitochondria; Short hairpin RNA

Introduction

Citrin is a calcium-binding mitochondrial solute-carrier protein primarily expressed in the liver, heart, and kidney. The human gene for citrin resides on chromosome 7q21.3 (SLC25A13) [1-3]. The mitochondrial aspartate-glutamate carrier (AGC) [4], which catalyzes a 1:1 exchange of aspartate for glutamate, is important in the malate/aspartate shuttle, urea synthesis, and gluconeogenesis from lactate [5]. Citrin is activated by calcium on the external side of the inner mitochondrial membrane [4].

Mutations in the human gene coding for citrin are responsible for adult-onset type II citrullinemia (CTLN2) [1,6], an autosomal recessive disease caused by a liver-specific deficiency in argininosuccinate synthetase. AGC plays an important role in the urea cycle in liver by providing aspartate for incorporation into argininosuccinate [7]. The citrin gene mutations in patients with CTLN2 are associated with either truncation of the protein or deletion of a loop between the transmembrane spans [1,6], resulting in functional impairment of citrin as an AGC in mitochondria. Such impairment could presumably block the supply of aspartate from mitochondria for argininosuccinate synthesis, with consequent alterations in the stability/activity of liver argininosuccinate synthetase, an abnormality seen in CTLN2.

CTNL2 is characterized clinically by episodic neurological symptoms associated with hyperammonemia, including disorientation, abnormal behaviors (aggression, irritability, and hyperactivity), seizures, coma, and potentially death from brain edema [8]. One of the clinical and pathological features of CTLN2 is the presence of fat deposition and fibrosis in liver [8-10]. Citrin deficiency impairs the

malate aspartate shuttle, which may increase cytosolic NADH and promote fatty degeneration and hyperlipidemia [8,9].

In the present study, we identified apoptotic hepatocytes during autopsy examination of a patient with CTLN2. We proposed that apoptosis of hepatocytes associated with the loss of AGC function might occur in CTLN2. Based on this finding, we further investigated the functional role of citrin in hepatocyte survival.

Materials and methods

Cells. Human hepatocellular carcinoma (HCC) cell lines SK-Hep-1, HuH-7, PLC/PRF/5, Hep3B, and HepG2 were used. These cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Human HCC cell lines SNU-423 and SNU-449 were also used, and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Liver tissue samples from a patient with CTLN2. A formalin-fixed liver sample obtained from a previously reported autopsy case [11] was embedded in paraffin wax for histopathological analysis. The 4-μm thick sections were examined after staining with hematoxylin and eosin or Azan stain. Apoptosis was assessed using the *In Situ* Apoptosis Detection Kit (Takara Bio Inc., Otsu, Japan). 3,3'-Diaminobezidine (Sigma Aldrich, St Louis, MO) staining identified apoptotic cells. Mutations in the SLC25A13 gene were detected by amplifying DNA fragments using the primers and conditions described previously [1,6]. Samples from the patient were used after obtaining written informed consent from the relatives. The Ethics Committee of the

Faculty of Medicine at the University of the Ryukyus approved this study, which was performed in accordance with the Declaration of Helsinki.

Determination of cell viability and apoptosis of cultured cells. Cell viability was determined by the WST-8 method (Cell Counting Kit-8, Wako Pure Chemical Industries, Osaka, Japan) based on the MTT assay as described previously [12]. Apoptosis was assessed by TUNEL assay using the ApopTag Red *In Situ* Apoptosis Detection Kit (Chemicon, Temecula, CA). Rhodamine-labeled anti-digoxigenin antibody was used for visualization of the apoptotic cells.

Detection of citrin mRNA by RT-PCR. Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using an RNA PCR Kit (Takara Bio Inc.). Thereafter, cDNA was amplified using 35 and 28 cycles for citrin and β-actin, respectively. The oligonucleotide primers used were as follows: citrin sense, 5’-GAAAGTGCTACGCTATGAAGG-3’ and antisense, 5’-GCAGTCTATCACTCCGCTGT-3’; and β-actin sense, 5’-GTGGGGCGCCCCAGGCACCA-3’ and antisense, 5’-CTCCTTAATGTCACGCACGATTTC-3’. The product sizes were 559 bp for citrin and 548 bp for β-actin. The thermocycling conditions for the targets were as follows: denaturing at 94°C for 60 s for citrin and for 30 s for β-actin, annealing at 53°C for 60 s for citrin and at 60°C for 30 s for β-actin, and extension at 72°C for 60 s for citrin and for 90 s for β-actin. The PCR products were analyzed by electrophoresis and ethidium bromide staining.

RNA interference. We selected four short-hairpin RNA (shRNA) targeted to cDNA sequences corresponding to positions 807-827, 1291-1311, 1311-1331, and 1389-1409 of the human citrin gene, and BLAST-confirmed them for specificity. Synthetic forward and reverse oligonucleotides were annealed respectively and cloned into the pRNAT-U6.1/Neo vector (GenScript, Piscataway, NJ) between the *Bam*HI and *Hind*III sites. Scrambled small-interfering RNA (siRNA) with no significant homology to mouse or human gene sequences were used as controls. These were designated using the "siRNA Target Finder" (Ambion, Austin, TX). The GFP marker under cytomegalovirus promoter control was used to monitor transfection efficiency. The recombinant plasmids were named pRNAT-citrin-siRNA-I, -II, -III, -IV, and pRNAT-Con. Hep3B cells were transfected using a MicroPorator (Digital Bio, Seoul, Korea), pulsed once at 1140 V for 50 ms.

Western blot analysis. Cells lysates were prepared and equal amounts of protein (20 μg) were subjected to electrophoresis on SDS-polyacrylamide gels, followed by transfer to a polyvinylidene difluoride membrane and sequential probing with the following antibodies: rabbit polyclonal anti-citrin [6], mouse monoclonal anti-PARP (BD Transduction Laboratories, San Jose, CA), rabbit monoclonal anti-cleaved caspase-3 (Cell Signaling, Beverly, MA), rabbit polyclonal anti-Bcl-xL (BD Transduction Laboratories), mouse monoclonal anti-Bcl-2 and anti-Bax (NeoMarkers, Fremont, CA), rabbit polyclonal anti-Bak (Cell Signaling), and mouse monoclonal anti-Hsp60 and anti-β-actin (NeoMarkes) as internal controls. The bands were

visualized with an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

Cytochrome c *release from mitochondria.* After cells were transfected, mitochondrial and cytosolic fractions were prepared using the Cytosol/Mitochondria Fraction Kit (Merck Biosciences, Darmstadt, Germany) according to the manufacturer's instructions. Cytochrome *c* was detected by Western blot analysis with a mouse monoclonal antibody to cytochrome *c* (Santa Cruz Biotechnology, Santa Cruz, CA), followed by chemiluminescence.

Immunohistochemical staining. Serial sections were deparaffinized in xylene and incubated with rabbit anti-citrin polyclonal antibody [6] for 1 h at room temperature. After washing with phosphate-buffered saline, the sections were incubated with biotinylated secondary antibody with avidin and biotinylated horseradish peroxidase complex (DAKO, Caterpillar, CA). 3,3'-Diaminobenzidine was used as a chromogen. Sections were finally counterstained with methyl green, hydrated in ethanol, cleaned in xylene, and mounted.

Caspase activity assay. Caspase-3/7 and caspase-9 activities were assessed using the Caspase-Glo^{TM} 3/7 and 9 Assay System (Promega, Madison, WI) using the instructions provided by the manufacturer. The reaction was analyzed by luminometry.

Results

A patient with CTLN2

This CTLN2 case was homozygous for mutation [I] (851del4), which is the second most common mutation in Japanese CTLN2 patients [9]. A 4-bp (GTAT) deletion from nucleotides 851 to 854 in exon 9 was observed. The autopsied liver showed marked fatty changes with severe fibrosis, namely cirrhosis (Fig. 1A and B). There was no evidence of inflammatory response or cholestasis, but a large number of hepatocytes were apoptotic (Fig. 1C) by TUNEL staining. Mutation [I] causes truncation of the citrin protein by frameshift, rendering the liver in these patients completely deficient in citrin [6]. Accordingly, immunostaining of liver sections with an anti-citrin antibody showed no citrin expression in hepatic cells of the CTLN2 patient compared with strong staining for citrin in the hepatocytes of controls (Fig. 1D and E). The finding of apoptotic hepatic cells in a CTLN2 case suggests a possible role for citrin in hepatocyte survival.

Expression of citrin in cultured cells

Using RT-PCR, we detected strong citrin mRNA expression in the liver and heart of a control individual, but only weak expression in the lung (Fig. 2A). Citrin mRNA was strongly expressed in HCC cell lines, SK-Hep-1, HuH-7, PLC/PRF/5, Hep3B, HepG2, and SNU-423. HCC cell line SNU-449 expressed lower levels of citrin mRNA. We also confirmed citrin expression in Hep3B at the protein level by immunohistochemical staining (Fig. 2B).

Knockdown of citrin protein expression

The shRNA expression vector that targets the citrin gene was used to assess the novel function of citrin in hepatocytes. Downregulation of citrin expression was achieved using four selected shRNA expression vectors that target the citrin gene. Each recombinant plasmid was tested for citrin-depletion efficiency. RT-PCR indicated that pRNAT-citrin-siRNA-III was the most efficient in suppressing citrin expression compared to Hep3B cells transfected with the control siRNA sequence (Fig. 3A). Thus, this sequence was used for further silencing experiments. The silencing specificity was confirmed by Western blot analysis of protein expression, which showed a significant decrease in citrin immunoreactivity in pRNAT-citrin-siRNA-III-transfected Hep3B cells compared with control cells (Fig. 3B). Actin was also blotted to confirm equal protein loading. The relative viability of cells transfected with control and pRNAT-citrin-siRNA-III was measured by WST-8 assay over time following transfection. At 12, 24, and 48 h post-transfection, the relative viability of Hep3B cells transfected with pRNAT-citrin-siRNA-III was significantly reduced (Fig. 3C).

Silencing the citrin gene by siRNA targeting induces apoptosis

Next, we evaluated the transfected cells for DNA fragmentation using TUNEL staining to address whether the cell death induced by citrin knockdown was apoptotic. Approximately 70% of Hep3B cells transfected with the citrin shRNA expression vector were TUNEL-positive compared with only 27% of control cells, indicating an increase in apoptosis in cells with downregulated citrin expression (Fig. 3D and E).

The TUNEL-positive population found in cells transfected with control shRNA is most likely explained by the presence of cells damaged by transfection procedure.

Citrin-related apoptosis is caspase-dependent

Western blot analysis further showed that citrin silencing induced PARP cleavage, a general sign of apoptosis, and caspase-3 cleavage (Fig. 4A). Silencing of the citrin gene was associated with four-fold increase in caspase-3/7 activity compared with cells transfected with control shRNA (Fig. 4B). Based on the above data, we suggest that inhibition of citrin expression resulted in Hep3B cell apoptosis, which proceeded with PARP cleavage and caspase-3/7 activation. Furthermore, we also demonstrated that citrin downregulation results in activation of caspase-9 (Fig. 4B). Apoptosis can occur via intrinsic and extrinsic pathways, with the former proceeding via caspase-9 activation [13]. Our results therefore suggest that downregulation of citrin activates the intrinsic pathway. Although the key component in both pathways is the activation of caspase-3 and caspase-7, the intrinsic pathway involves mitochondria and Bcl-2-family proteins. More than 20 Bcl-2 family members have been identified, with some being antiapoptotic including Bcl-2, Bcl-xL, and Bcl-W, while others are proapoptotic such as Bax and Bak [13]. To clarify the pathway through which citrin knockdown induced apoptotic cell death in this study, we measured Bcl-2, Bcl-xL, Bax, and Bak expression in our cells. As shown in Fig. 4A, Bcl-2 and Bcl-xL were downregulated by citrin siRNA, while Bax and Bak were upregulated.

Citrin knockdown induces release of cytochrome c *from mitochondria*

Studies have shown that the involvement of mitochondria in apoptosis includes a critical step involving the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol [14]. To test if downregulation of citrin protein levels by siRNA induced cytochrome *c* efflux, we isolated mitochondrial and cytosolic fractions (postmitochondrial supernatants) from citrin shRNA expression vector-transfected Hep3B cells and control cells and analyzed the levels of cytochrome *c* by Western blotting. A strong cytochrome *c* immunoreactivity was detected in the cytosolic fraction prepared from citrin shRNA expression vector-transfected Hep3B cells by comparison with controls (Fig. 4C), indicative of its release from the mitochondrial intermembrane space to the cytosol. In contrast, cytochrome *c* was confined to mitochondria in cells transfected with the control shRNA (Fig. 4C). The levels of actin and Hsp60 (used as loading controls for the cytoplasm and mitochondria, respectively) were comparable in all samples analyzed. These results demonstrate that downregulation of citrin protein expression induced the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol.

Discussion

Citrin deficiency induces two clinical features: neonatal intrahepatic cholestasis caused by citrin deficiency and CTLN2 [1-3]. Citrin is an AGC located on the inner membrane of mitochondria that plays a role in the malate-aspartate NADH shuttle and urea synthesis. Citrin deficiency leads to an increased $NADH/NAD^+$ ratio in the cytosol and the failure of aspartate supply from the mitochondria to the cytoplasm for synthesis of arginosuccinate in the arginosuccinate synthetase reaction. These effects

result in hypercitrullinemia and hyperammonemia [5,9]. However, little is known about the potential contribution of citrin to hepatocyte homeostasis. Here we showed that hepatic cells of a patient with CTLN2 exhibited apoptosis with fat deposition and fibrosis. We hypothesized that mitochondrial citrin plays an important role in regulating hepatocyte survival through control of apoptosis. Because evidence suggests that fatty acid accumulation in non-adipose cells is associated with apoptosis [15], we cannot exclude the possibility that fatty acids may play a relevant role in apoptosis. Although further *in vivo* studies are needed to confirm the result, it is difficult to analyze because of the low incidence of CTLN2 in Japan (1/100,000 to 1/230,000) [16,17]. We then assessed the physiological role of citrin function in hepatic cells using Hep3B cells, since citrin is abundantly expressed in this cell line.

RNA interference is a promising new experimental tool for gene function analysis, and siRNA vectors can be transfected into mammalian cells by various means. First, chemically synthesized siRNA can be introduced into cells [18], although variable transfection efficiencies and the transient nature of silencing by exogenously delivered siRNA limit the applicability of this approach [19]. The use of shRNAs circumvents many of these limitations. The transcribed shRNA from transfected plasmids is processed to siRNAs by the intrinsic cellular machinery to achieve stable RNA interference. Studies using different cell types and shRNAs against various genes have demonstrated the efficacy of endogenously expressed shRNA [20].

In the present study, we used shRNAs that targeted citrin to silence the expression of citrin in Hep3B cells. Of the four shRNAs originally selected, our

results demonstrated that recombinant plasmid pRNAT-citrin-siRNA-III was the most effective in suppressing citrin expression, and that citrin knockdown induced apoptosis of Hep3B cells.

The siRNA-mediated downregulation of citrin protein expression in Hep3B cells activated caspase-3/7, caspase-9, and PARP, suggesting that knockdown of citrin triggers the intrinsic mitochondrial pathway of apoptosis. The release of cytochrome *c* from the mitochondrial intermembrane space to the cytosol is an essential event of mitochondrial cell death [21]. Regardless of the molecular mechanism of cytochrome *c* release, once in the cytosol, cytochrome *c* will induce caspase activation and apoptotic cell damage [21]. Cytochrome *c* was released from mitochondrial intermembrane space to the cytosol in Hep3B cells transfected with the citrin shRNA expression vector. Depletion of citrin also enhanced the expression of apoptosis-promoting factors, Bax and Bak, and inhibited the expression of antiapoptotic proteins, Bcl-2 and Bcl-xL. Bcl-2 family members are the principal regulators of the mitochondrial-dependent pathway for apoptosis [13], further supporting a role for citrin in this pathway. Bcl-2 proteins localize to the outer membrane of the mitochondria and alter membrane potential, possibly by interacting with the permeability transition pore [22]. However, citrin localizes to the inner membrane of the mitochondria. We therefore cannot speculate from our results on the molecular mechanism leading to apoptotic cell death of hepatic cells caused by citrin knockdown, and further investigations are required.

Aralar is the second human AGC isoform [5]. It is known that aralar is expressed mainly in skeletal muscle, heart, and brain, but not in liver [23]. However,

aralar mRNA was expressed in all HCC cell lines except for HuH-7 (data not shown). We examined whether knockdown of aralar could affect the viability of Hep3B cells. Unlike citrin, depletion of aralar did not reduce the cell viability (data not shown), suggesting that the role of citrin and aralar in cell survival is different.

In conclusion, the present study indicated that siRNA targeting of citrin mRNA via a plasmid-based system effectively sustains knockdown of citrin gene expression in Hep3B cells. The impaired citrin expression led to Hep3B cell apoptosis through upregulation of Bax and Bak, and downregulation of Bcl-xL and Bcl-2. Our findings demonstrate a novel role for citrin as a negative regulator of the mitochondrial-dependent pathway mediating apoptosis.

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Figure Legends

Fig. 1 Histopathology of a patient with CTLN2. (A) Cirrhotic changes with fatty deposition (hematoxylin and eosin, ×100). The cytoplasm of the liver cells was filled with lipid droplets of various sizes. (B) Severe fibrosis was also observed (Azan stain, ×100). (C) TUNEL assay showed apoptosis in the hepatocytes. Immunohistochemical expression of citrin on liver sections from a CTLN2 patient (D) and a control (E) (×100).

Fig. 2 Expression of citrin in liver and HCC cell lines. (A) Expression of citrin mRNA in various human tissues and HCC cell lines. Representative results of three similar experiments are shown. (B) Citrin expression in Hep3B cells was examined by immunostaining (×100).

Fig. 3 Downregulation of citrin induces apoptosis. (A) RNA extracted from Hep3B cells transfected with the indicated shRNA expression vector were subjected to RT-PCR for citrin and β-actin mRNA. β-actin expression served as a control. (B) Cell lysates were prepared from Hep3B cells transfected with pRNAT-Con and pRNAT-citrin-siRNA-III, and immunoblotted with an anti-citrin antibody. Actin was also immunoblotted as a loading control. Representative results of three similar experiments are shown. (C) Inhibition of cell viability by citrin shRNA expression vector. (D) TUNEL staining of Hep3B cells transfected with pRNAT-citrin-siRNA-III. The majority of Hep3B nuclei of cells depleted of citrin were TUNEL-positive.

Hep3B cells transfected with the control shRNA showed little TUNEL-positive staining. (E) Quantification of the proapoptotic effect of citrin knockdown, using GFP as a cell marker. Data of (C) and (E) are mean ± SD of three independent experiments.

Fig. 4 Citrin downregulation induces apoptosis of Hep3B cells through the mitochondrial death pathway. (A) Effect of citrin siRNA on cleavage of PARP and caspase-3, and on expression of Bcl-2 family proteins. Total cell lysates from Hep3B cells transfected with the control and pRNAT-citrin-siRNA-III vectors were separated electrophoretically on polyacrylamide gels and immunoblotted with an antibody against each protein, and actin as an internal control. Representative results of three similar experiments in each panel are shown. (B) Measurement of caspase-3/7 and caspase-9 activities. The results are mean ± SD of three independent experiments. (C) shRNA-mediated downregulation of citrin induces release of cytochrome *c* from mitochondria. Equal amounts of cytosol and mitochondrial proteins isolated from Hep3B cells transfected with the control and pRNAT-citrin-siRNA-III vectors were subjected to SDS-polyacrylamide gels and immunoblotted with anti-cytochrome *c* antibody. Representative results of three similar experiments are shown.