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Mitochondrial iron accumulation exacerbates hepatic toxicity caused by hepatitis C virus core protein

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ABSTRACT

Patients with long-lasting hepatitis C virus (HCV) infection are at major risk of hepatocellular carcinoma (HCC). Iron accumulation in the livers of these patients is thought to exacerbate conditions of oxidative stress. Transgenic mice that express the HCV core protein develop HCC after the steatosis stage and produce an excess of hepatic reactive oxygen species (ROS). The overproduction of ROS in the liver is the net result of HCV core protein-induced dysfunction of the mitochondrial respiratory chain. This study examined the impact of ferric nitrilacetic acid (Fe-NTA)-mediated iron overload on mitochondrial damage and ROS production in HCV core protein-expressing HepG2 (human HCC) cells (Hep39b cells). A decrease in mitochondrial membrane potential and ROS production were observed following Fe-NTA treatment. After continuous exposure to Fe-NTA for six days, cell toxicity was observed in Hep39b cells, but not in mock (vector-transfected) HepG2 cells. Moreover, mitochondrial iron (^{59}Fe) uptake was increased in the livers of HCV core protein-expressing transgenic mice. This increase in mitochondrial iron uptake was inhibited by Ru360, a mitochondrial Ca^{2+} uniporter inhibitor. Furthermore, the Fe-NTA-induced augmentation of mitochondrial dysfunction, ROS production, and cell toxicity were also inhibited by Ru360 in Hep39b cells. Taken together, these results indicate that Ca^{2+} uniporter-mediated mitochondrial accumulation of iron exacerbates hepatocyte toxicity caused by the HCV core protein.

Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease. About 120–200 million people are infected with HCV, increasing their risk of developing chronic hepatitis, cirrhosis, and eventually hepatocellular carcinoma (HCC) (Ikeda et al., 1998; Nishioka et al., 1991). The HCV genome is approximately 9.6 kb in size and encodes a polyprotein of ~3000 amino acids. The large HCV polyprotein is cleaved by host and viral proteases to generate at least ten smaller proteins, including four structural proteins (one core protein, two envelope proteins, and the E1, E2, and p7 ion channels) (Bukh et al., 1995) and six non-structural proteins (NH2-NS2, NS3, NS4A, NS4B, NS5A, and NS5B-COOH) (Bartenschlager and Lohmann, 2000). These proteins participate in viral replication and also influence cellular functions of the host.

Oxidative stress is commonly observed following HCV infection and is caused by increased levels of cellular reactive oxygen species (ROS) or by changes in cellular antioxidant capacities (Choi and Ou, 2006; Oberley, 2002; Otani et al., 2005). In particular, HCV core protein is known to be closely associated with the mitochondria and causes the increase in host ROS production, lipid peroxidation (Lau et al., 1998; Moriya et al., 2001; Okuda et al., 2002) and mitochondrial Ca^{2+} uptake. HCV core protein also reduces GSH and NADPH concentrations and mitochondrial complex I activities. These undertakings ultimately disrupt mitochondrial membrane permeability and trigger mitochondrial dysfunction (Wang et al., 2010; Wang and Weinman, 2006). As mitochondrial function is the master regulator of cellular energy and apoptotic cell death, mitochondrial injury can culminate in metabolic deficits and steatohepatitis, further exacerbating cell injury and dysfunction.

Due to the relationship between chronic HCV infection and the development of HCC, numerous studies have attempted to identify the HCV proteins that are responsible for hepatocarcinogenesis. These studies indicate that the HCV core protein can promote the immortalization of primary human hepatocytes (Ray et al., 2000), whereas the non-

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; ROS, reactive oxygen species; Fe-NTA, ferric nitrilacetic acid; JC-1, 5,5′,6,6′-tetrachrolo-1,1′,3,3′-tetraethylbenzimidazoly-carbocyanine iodide; CCCP, carbonyl cyanide-m-chlorophenyl hydrazine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HPF, hydroxyphenyl fluorescein; ANT, adenine nucleotide translocator; HRP, horseradish peroxidase; DMEM, Dulbecco's Modified Eagle's Medium; CL, chemiluminescence; TTBS, Tris-buffered saline/0.05% Tween 20; BSA, bovine serum albumin; Hep39b, HCV core protein-expressing HepG2; Hepswx, vector-transfected HepG2.

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structural proteins NS3 and NS4B can transform NIH 3T3 cells, either individually or in combination with Ha-ras (Park et al., 2000). Iron overload in the liver, which is associated with the genetic disorder hereditary hemochromatosis, has been thought to increase the risk of HCC by about 200-fold (Bonkovsky et al., 1997; Kowdley, 2004). For example, the livers of patients afflicted with HCV are characterized by the elevated expression of transferrin receptor 1 and hepcidin, both of which stimulate iron uptake into hepatocytes (Bonkovsky et al., 1997; Hayashi et al., 1994). In contrast, iron depletion (in the form of dietary iron restriction and/or phlebotomy) can improve hepatic inflammation and lower serum aminotransferase activity in HCV patients (Hayashi et al., 1994). Thus, a major precipitating factor for the pathogenesis of HCV-related liver disease has been attributed to the augmentation of oxidative stress following iron accumulation. However, no underlying cellular mechanism has yet been elucidated.

This study investigated the effect of iron exposure on mitochondrial dysfunction, ROS production and cell toxicity in human hepatoma cells stably expressing the HCV core protein (Hep39b cells). The underlying mechanism responsible for mitochondrial iron accumulation in Hep39b cells and in the livers of HCV core protein-expressing transgenic mice was also examined.

Materials and methods

Chemicals and reagents. Ferric nitrate nonahydrate, nitrilotriacetic acid (NTA), 5,5′,6,6′-tetrachrolo-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), carbonyl cyanide-m-chlorophenyl hydrazine (CCCP) and G418 disulfate were from Sigma Aldrich (St. Louis, MO). MitoTracker® Red was from Invitrogen (Carlsbad, CA). $^{59}FeSO_4$ was from Perkin-Elmer (Waltham, MA). Ru360 was from Merck Millipore Japan (Tokyo, Japan). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hydroxyphenyl fluorescein (HPF) was from Sekisui Medical Co., Ltd. (Tokyo, Japan). Adenine nucleotide translocator (ANT) goat polyclonal IgG, CCDC109A goat polyclonal IgG and horseradish peroxidase (HRP)-conjugated anti-goat IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All chemicals and solvents were of analytical grade.

Preparation of Fe-NTA. The Fe-NTA complex was prepared as described by Awai et al. (1979). Briefly, ferric nitrate was dissolved in 1 N HCl to form a 50 mM solution, and NTA was dissolved in 1 N NaOH to form a 150 mM solution. Equal volumes of the two solutions were mixed just before the experiment, and the pH was adjusted to 7.4 with $NaHCO_3$.

Assessment of cytotoxicity. Cytotoxicity was assessed by the MTT assay. Briefly, Hep39b and Hepswx cells were seeded into 96 well culture plates at a density of 8.4×10^3 cells/well and were exposed to various concentrations of Fe-NTA the following day, the medium was replaced with fresh medium containing the same component every 24 h. In some conditions, cells were treated with 20 μM Ru360, a mitochondrial Ca^{2+} uniporter inhibitor, for 1 h prior to Fe-NTA exposure. After six days, the cell culture medium was replaced by 50 μl MTT solution (5 mg/ml MTT in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM)), and the cells were incubated for 2 h at 37 °C. To dissolve the reduced MTT crystals, 200 μl isopropanol was added. The absorbance of the dye was measured at a wavelength of 570 nm, and the turbidity of the cells (background absorbance) was measured at a reference wavelength of 630 nm. The absorbance of the controls (Hepswx and Hep39b) was set at 100%, and cytotoxicity was calculated as the absorbance of the experimental sample relative to that of the control.

Assessment of ROS production. ROS production was first assessed by chemiluminescence (CL) analysis. Briefly, cells were seeded into 35 mm glass-bottomed dishes at a density of 2.5×10^5 cells/dish and exposed to 300 μM Fe-NTA the following day, the medium was replaced with fresh medium containing the same component every 24 h. In some cases, cells were treated with Ru360 for 1 h prior to Fe-NTA treatment. After five days, the cell culture medium was replaced with phenol red-free DMEM containing Fe-NTA and Ru360, and the dish was protected from light. The following day, spontaneous CL was measured using a single photoelectron counting system (CLD-10; Tohoku Electronic Industrial Co., Ltd., Sendai, Japan), as described previously (Maeda et al., 2010). Emission was expressed in counts/10 min/mg protein.

ROS production was also assessed using HPF as a fluorescent probe for the selective detection of hydroxyl radicals. Briefly, cells were seeded into 35 mm glass-bottomed dishes, as described for CL analysis. After 7 days, the cell culture medium was replaced with modified Hanks' balanced salt solution (HBSS) containing 10 mM HEPES, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 2.7 mM glucose (pH 7.4). Next, 10 μM HPF and 20 nM MitoTracker® Red (a fluorescent probe for the mitochondria) were added, and cells were incubated for 15 min at 37 °C. Images of HPF and MitoTracker® Red staining were obtained using a laser scanning confocal microscope (FV300; Olympus Optical Co., Ltd., Tokyo, Japan). The wavelengths (excitation/emission) for the detection of HPF (green) and MitoTracker® Red (red) were 488 nm/515 nm and 579 nm/599 nm, respectively.

Assessment of mitochondrial membrane potential. Measurement of mitochondrial membrane potential was performed using the JC-1 stain, a lipophilic cation fluorescent dye that accumulates in the mitochondria. At a low mitochondrial membrane potential, the JC-1 dye exists as a monomeric molecule and fluoresces green, whereas at a higher membrane potential the JC-1 dye forms polymeric aggregates and fluoresces red. A fall in the mitochondrial membrane potential is therefore indicated by a decrease in the ratio of red signal to green signal.

Cells were cultured in 96 well black culture plates at a density of 8.4×10^3 cells/well and exposed to various concentrations of Fe-NTA the following day, the medium was replaced with fresh medium containing the same component every 24 h. After six days, the culture medium was replaced with 200 μl JC-1 solution (10 μg/ml JC-1 in HBSS), and cells were incubated in the dark for 30 min at 37 °C. After washing twice with HBSS, the absorbance of the cells in each well was immediately measured using a fluorescence plate reader with the excitation and emission wavelengths set at 490 nm and 530 nm (green)/590 nm (red), respectively.

Animals. The production of transgenic mice expressing the HCV core protein has been described previously (Moriya et al., 2001). Briefly, the HCV core protein gene was placed downstream of a transcriptional regulatory region from the hepatitis B virus and introduced into C57BL/6 mouse embryos (Clea Japan, Tokyo, Japan). All of the animals were treated humanely in accordance with the guidelines issued by the National Institute of Health and all procedures described below were approved by the animal care committee of Chiba University.

Isolation of mouse liver mitochondria. The mouse liver mitochondrial fraction was prepared according to a previously described method (Masubuchi et al., 2002). Livers were isolated from two mice and placed in ice-cold buffer containing 250 mM sucrose, 10 mM HEPES–KOH, and 0.5 mM EGTA (pH 7.4). Livers were cut into small cubes with scissors in the same buffer and homogenized five times with a Potter homogenizer. The homogenates were diluted to 0.25 g liver/ml and were centrifuged at 770 ×*g* for 5 min at 4 °C. The resulting supernatant was decanted and further centrifuged at 9800 ×*g* for 10 min. The pellet was resuspended to yield a concentration of 0.5 g liver/ml in buffer containing 250 mM sucrose, 10 mM HEPES–KOH and 0.3 mM EGTA (pH 7.4), and centrifuged at 4500 ×*g* for 10 min. The pellet was resuspended to yield a concentration of 1 g liver/ml in the same buffer and centrifuged at 2000 ×*g* for 2 min, followed by further centrifugation at 4500 ×*g* for 8 min. The