Free Radical Biology & Medicine 51 (2011) 1806–1814

Contents lists available at SciVerse ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Occult hepatitis C virus elicits mitochondrial oxidative stress in lymphocytes and triggers PI3-kinase-mediated DNA damage response

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ARTICLE INFO

Article history:
Received 27 May 2011
Revised 11 August 2011
Accepted 12 August 2011
Available online 3 September 2011

Keywords:
mtDNA
DNA repair
Apoptosis
Circulating nucleosomes
Translational research
Free radicals

ABSTRACT

Occult hepatitis C viral infection (OHCI) is a newly reported pathological entity associated with increased risk of developing hepatocellular carcinoma and lymphoproliferative disorders. Although hepatocytes are the primary sites of viral replication, hepatitis C virus is potentially lymphotropic, invading and propagating in cells of the immune system. Lymphocytes, the extrahepatic viral reservoirs, are differentially implicated in the occult and the active forms of the disease. This study aimed to elucidate the implications of mitochondrial oxidative stress on the immune pathophysiological mechanisms of OHCI. We herein report that OHCI induces mitochondrial oxidative stress, leading to DNA double-strand breaks and elicitation of a phosphoinositol 3-kinase-mediated cellular response in peripheral blood lymphocytes. Compared to controls, OHCI subjects showed higher accumulation of pATM, pATR, γH2AX, and p-p53, along with active recruitment of repair proteins (Mre11, Rad50, and Nbs1) and altered mitochondrial DNA content. Increased mitochondrial membrane depolarization and circulating nucleosome levels along with chromatid-type aberrations and decreased T-cell proliferative index observed in the OHCI group further indicated that this damage might lead to Bax-triggered mitochondria-mediated cellular apoptosis. Together our results provide the mechanistic underpinnings of mitochondrial dysfunction in OHCI, a previously unknown paradigm, for explaining the immune pathogenesis in a redox-dependent manner.

Hepatitis C virus (HCV) infection is prevalent in approximately 2% of the world's population and is one of the leading causes of cirrhosis, hepatocellular carcinoma, and lymphoproliferative disorders, including mixed cryoglobulinema and non-Hodgkin B cell lymphoma [1]. HCV, a sole member of the genus *Hepacivirus* (family Flaviridae), is a positive-strand RNA virus that replicates by synthesizing a negative RNA strand of 9600 bp [2]. The viral RNA encodes a large polyprotein of 3100 amino acids, which is translated on the endoplasmic reticulum (ER) and posttranslationally processed by cellular and viral proteases into 10 individual proteins. The structural proteins (core, E1, and E2) build up the virus particle, whereas the p7 polypeptide and the nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are required for RNA replication and virion assembly [3]. The nonstructural proteins orchestrate viral replication, forming a membrane-associated replication complex. All HCV proteins exposed to the cytosolic space are anchored to the ER membrane, except E1 and E2, which face the ER lumen [4]. Because HCV has no known ability to integrate its nucleotide sequence into the host genome and has too short a half-life, an uninterrupted HCV replication is essential to maintaining a continuous infectious state.

Evidence derived from experimental systems illustrate the strong association of viral replication with mitochondrial oxidative stress and generation of reactive oxygen species (ROS) [5]. Oxidative stress imposed either directly by the virus or by the host immune response is considered an important pathogenic mechanism in HCV infection. Viral proteins such as HCV core, E1, and NS3 are reported to be potent inducers of ROS [6]. These proteins inactivate mitochondrial respiratory chain enzymes and trigger blockade of the electron chain, dissipation of the mitochondrial transmembrane potential, and increased electron leakage causing hypergeneration of endogenous ROS [7]. Although the synthesis and maturation of HCV proteins occur at the level of the ER,

Abbreviations: 8-oxo-dG, 8-oxo-2′-deoxyguanosine; ALT, alanine transferase; ARP, aldehyde-reactive probe; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester; DCF, 2′,7′-dichlorofluorescin; DSB, double-strand break; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; GR, glutathione reductase; HCV, hepatitis C virus; OHCI, occult hepatitis C infection; pATM, ataxia telangiectasia mutated phosphoserine-1981; pATR, ataxia telangiectasia and Rad3-related phosphoserine-428; p-p53, p53 phosphoserine-15; PE, phycoerythrin; PCR, polymerase chain reaction; PI3-kinase, phosphoinositol 3-kinase; ROS, reactive oxygen species; SOD, superoxide dismutase; SVR, sustained virological response.

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doi:10.1016/j.freeradbiomed.2011.08.009

studies have shown that HCV proteins accumulate at the point of contact between the mitochondrial outer membrane and the ER. The viral proteins have been speculated to migrate from the ER to the mitochondria by lateral diffusion via transient fusion of the membranous subcompartments [8]. Therefore, direct interaction of HCV proteins with the mitochondrial machinery in hepatic and extrahepatic sites is posited, although the mechanistic insights of this interaction remain obscure.

Despite the fact that hepatocytes are the primary sites of virus replication, HCV is potentially lymphotropic. HCV invades and propagates in cells of the immune system, albeit at a lower rate per cell than hepatic tissue [9]. Indeed, HCV RNA positive and negative (replicative) strands as well as virus proteins have been identified in peripheral blood lymphocytes of chronic HCV patients [10,11]. HCV lymphotropism is also supported by the presence of low levels of viral RNA in circulating lymphocytes of occult HCV patients, even after apparent resolution of the disease [12]. As lymphocytes, the viral reservoirs, are differentially implicated in the occult and the active forms of the disease, the possible molecular repercussions of viral interaction at the subcellular level need to be determined.

This work is an extension of our previous study in which we observed sustained virological response (SVR) in 94.3% of genotype 3-infected patients after combination therapy [13]. SVR is defined as undetectable HCV RNA in serum, even after 24 weeks withdrawal of standard combination therapy (PEGylated interferon and ribavirin) [14]. The incidence of secondary occult HCV infections has been identified in cases that achieved SVR. Further, we aimed to understand the implications of viral interaction with the mitochondrial machinery of the host by evaluating downstream cellular effects. Oxidative stress, DNA damage repair response, mitochondrial DNA (mtDNA) content and respiratory chain enzyme activity, T-cell proliferative index, and relative expression of proapoptotic Bax along with cytogenetic analysis and apoptosis in the peripheral blood lymphocytes of occult and chronic HCV and control subjects were studied.

Materials and methods

Subject selection

A total of 68 chronic HCV patients were treated with standard combination therapy of PEGylated interferon and ribavirin for 24 to 48 weeks. Of these, 53 patients infected with genotype 3 achieved SVR [13]. The presence of HCV RNA was observed in peripheral blood lymphocytes in 10 (of 53) patients negative for serum HCV RNA but with normal liver function, thereby confirming secondary occult HCV infection. EDTA-conjugated blood samples were collected from each subject by routine venipuncture and used for investigations. Diagnosis of chronic HCV ($n=30$) was made on the basis of a history of more than 6 months of liver disease together with a positive HCV antibody test (second-generation enzyme immunoassay) and detectable HCV RNA by real-time PCR. The alanine transferase (ALT) levels of these patients were also raised on at least two determinations performed within 6 months and liver biopsy findings were consistent with chronic HCV within 18 months before therapy. There was no serologic evidence of co-infection with other hepatotropic viruses. Control subjects ($n=30$) recruited for the study were without any clinical history of hepatitis and considered healthy after routine laboratory analysis. Other possible associations of cellular injury, such as co-infection or consumption of tobacco, alcohol, or drugs, were cause for exclusion. All investigations were conducted as per institutional review board guidelines.

Lymphocyte isolation and culture

Lymphocytes were isolated by density gradient centrifugation using Lymphosep (MP Biomedicals, Solon, OH, USA) and viability was examined using the trypan blue dye exclusion test. Cells (1×10^6) were cultured and mitogenically stimulated as reported elsewhere [15].

Detection of HCV RNA

HCV RNA was isolated using the QIAamp viral RNA mini extraction kit (Qiagen, Hilden, Germany) following the protocol described previously by Bhargava et al. [16]. Briefly, viral particles were lysed under highly denaturing conditions followed by binding of viral RNA to a silica gel-based membrane and elution using RNase-free buffer. Detection of HCV RNA was done using LightCycler 2.0 real-time PCR (Roche Applied Sciences, Mannheim, Germany) [13].

Evaluation of oxidative stress

Intracellular ROS generation was measured from the percentage of 2′,7′-dichlorofluorescin (DCF) fluorescence using 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) labeling (Molecular Probes, Invitrogen, Carlsbad, CA, USA) as described elsewhere [17]. Quantitative ELISA for the oxidative stress biomarker 8-oxo-2′-deoxyguanosine (8-oxo-dG) and antioxidant enzymes glutathione reductase (GR) and superoxide dismutase (SOD) (Trevigen, Gaithersburg, MD, USA) was performed as per the manufacturer's instructions and optical density was measured at 450 and 340 nm [18].

Oxidative DNA damage quantification

Briefly, genomic DNA was isolated from the Wizard DNA purification kit (Promega) and dissolved in TE buffer at 100 μg/ml concentration. Formation of aldehyde-reactive probe (ARP) sites in each sample was quantified in accordance with the supplier's manual and absorbance was measured at 650 nm on an ELISA reader [19].

Mitochondrial enzyme activity

Measurement of enzyme activity, expressed as nmol/min/mg lymphocyte protein, of mitochondrial respiratory chain individual complexes was performed spectrophotometrically at 37 °C in a cuvette containing 1 ml of medium. We evaluated complex II (succinate ubiquinone reductase), complex III (ubiquinol–cytochrome *c* reductase), and complex IV (cytochrome *c* oxidase) enzyme activities following the method of Rustin et al. [20].

Relative quantitation of mitochondrial DNA/nuclear DNA ratio

Quantification of mtDNA in each subject was done through real-time PCR, with modifications [21]. In brief, two pairs of primers were designed and used in the two steps of relative quantification for mtDNA content: one pair for the amplification of the mitochondrial NADH dehydrogenase ortholog 1 (MT-ND1) gene (ND1-F, 5′-CCCTAA AACCCGCCACATCT-3′; ND1-R, 5′-GAGCGATGGTGAGAGCTAAGGT-3′) in mtDNA and another for the amplification of the single-copy nuclear gene human β-actin from the TIB MOLBIOL Universal Probe Library (Berlin, Germany). The data were expressed as the ratio of the mean mtDNA value of the triplicate measurements to the mean nDNA value (β-actin) of the triplicate measurements for a given sample (mtDNA/β-actin).

Assessment of DNA damage response

Immunofluorescence analysis was performed in all healthy controls ($n=30$), chronic patients ($n=30$), and patients with occult infections ($n=10$). Lymphocytes were fixed in 10% formaldehyde for 1 h, permeabilized with 0.1% Triton X-100 for 30 min, blocked with 3% bovine serum albumin for 3 h, and incubated with primary antibodies against