Escalated Expression of Autophagy Related Gene Atg5 in Hepatitis B Virus Infection

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ABSTRACT

Autophagy is a crucial catabolic process by which cells remove long-lived proteins and damaged organelles for recycling. Viral infections may also induce autophagic response. Members of autophagy related genes (Atgs) play active role in regulation of autophagic machinery. Atg5 gene is a prominent candidate responsible for initiation of autophagic process through the formation of autophagosome. Hepatitis B virus (HBV) has been shown to induce autophagy in experimental conditions. Since the expression status of Atg5 has not been elucidated in HBV infected cells from patients, we studied the same in peripheral blood mononuclear cells (PBMCs) of HBV infected subjects. Forty two HBV infected subjects were appraised for their status of infection by biochemical, serological and real time polymerase chain reaction (PCR) assay. PBMC derived total RNA were subjected to reverse transcription PCR for amplification of Atg5 mRNA. HBV pregenomic RNA (pgRNA) was also assessed as a marker of intracellular viral replication. PBMCs from 24 healthy voluntary blood donors were used as control cells. In contrast to controls, expression of Atg5 was significantly (p=0.0001) up regulated in infected PBMCs obtained from patients. Considering the role of Atg5 expression in viral replication, the present study is indicative of HBV mediated modulation of host’s autophagy machinery to establish a chronic infection.

Keywords: Hepatitis B virus, Peripheral blood mononuclear cells, Atg5 gene, Autophagy, Gene expression

INTRODUCTION

Hepatitis B virus (HBV) is a major causative agent of chronic liver diseases (CHB) having concomitant upshot of liver cirrhosis and hepatocellular carcinoma (HCC) with an approximate mortality of 1 million infected persons per year (Okuda. 2000). The variable outcome of CHB has been attributed to the virus induced modulation of host cellular components. This has also been related to viral clearance as well as cell mediated immune response (Wang et al., 2003). Thus, the interplay of the viral replication aptitude and the retaliatory host immune response is an imperative seminal issue of the odds of progressive liver disease (Rehermann. 2003). HBV, which is primarily hepatotropic and secondarily lymphotropic in human, possess a 3.2-kb partially double-stranded circular DNA genome having four open reading frames (ORFs) i.e. S, C, P, and X genes. While the first three ORFs code for the surface antigens, core/precore proteins, and the viral DNA polymerase, the X gene is responsible for generation of multifunctional regulatory proteins. One of the important replication intermediate is termed pregenomic RNA (pgRNA) that can be converted to the viral genome through the action of viral DNA polymerase which also acts as a reverse transcriptase.
Autophagy is an evolutionarily conserved intracellular homeostatic process that help in the clearance of unused/excess cytosolic macromolecules and damaged sub cellular organelles through lysosomal degradation and recycling (Kirkgaard, 2009). A defective autophagy pathway has allusion in a rising number of human diseases like infectious diseases, neuronal disintegrative disorders and cancer (Mizushima et al., 2008). Apart from its role in maintaining intracellular homeostasis in mammalian cells, autophagy functions as an important host defense mechanism concerned in innate and adaptive immune responses to the microbial infections (Schmid et al., 2006). The autophagy related gene (Atg) encoded proteins and autophagy machinery play key roles in regulating both positive and negative anti viral immune responses. One of the subset of Atgs, the Atg5, well thought-out to be involved in initiation of autophagosome precursor synthesis, is also considered to be an important mediator of apoptosis. Upon generation of death stimuli, Atg5 can undergo cleavage and the cleaved product appears to espouse mitochondria mediated apoptosis (Luo and Rubinsztein, 2007).

During viral infection, many cellular processes are either circumvented or destabilized as modulated by the virus. It has been suggested that in viral infections, the autophagy system can mortify viruses by transporting viral antigens to endosomes containing Toll-like receptors, and bringing antiviral proteins to viral replication sites (Yordi et al., 2013). A number of viruses have been shown to either suppress or induce autophagy for their survival against host degradation machinery or to benefit their replication using the autophagic membrane vesicles (Jackson et al., 2005; Prentice et al., 2004).

In cell culture studies, HBV has been shown to augment autophagy without mounting autophagic protein degradation and such induction of autophagic response was correlated with increased replication of viral DNA (Sir et al., 2010). Furthermore, using HBV transgenic mice with liver-specific knockout of the Atg5 gene, (Tian et al, 2011)) showed that HBV induced initiation of autophagy through mediation of Atg5 is crucial and beneficial for the virus to enhance its replication. In vitro cell culture and transfection experiments further corroborated the role of autophagy in HBV infection where the host’s autophagy system is galvanized during HBV infection to boost HBV replication (Li et al., 2011).

Despite the available experimental data regarding induction of autophagy by HBV in cell cultures, informations about the occurrence of similar phenomenon in HBV infected patients are still wanting. The present study was therefore conducted to evaluate the expression profile of Atg5 gene as a marker of initiator of autophagy in HBV infected peripheral blood mononuclear cells (PBMCs) obtained from CHB subjects. The presence of pgRNA as replication intermediate of HBV has been used as a marker for the presence of replicating virus in infected PBMCs obtained from patients.

MATERIALS AND METHODS

Patients

Based upon the clinical history, presentations, imaging records and serological status, 42 subjects who appeared positive for HBV surface antigen (HBsAg) were included in the study. Co-infection with human immunodeficiency virus (HIV), hepatitis A virus (HAV), hepatitis C virus (HCV), hepatitis delta virus (HDV) or hepatitis E virus (HEV) formed the basis of exclusion of HBV infected subjects from this study. A cluster of 24 healthy voluntary blood donors dished up as controls. Informed consents were taken from all the subjects prior to study and the study protocol was approved by the institutional ethics committee.

Serologic and biochemical parameters

Serum alanine transferase (ALT) and aspartate transferase (AST) levels were estimated by an automated clinical biochemistry analyzer (Randox, Oceanside, CA). HBV e antigen (HBeAg) and antibody (anti- HBe) status in sera of patients were determined by commercial enzyme-linked immunosorbent assay (ELISA) kits (Amar-EASE, Taiwan) as per the manufacturer’s directives.

HBV DNA quantitation by real time polymerase chain reaction (PCR)

Extraction of HBV DNA from patient’s sera was carried out using the High Pure SystemViral Nucleic Acid Kit (Roche Molecular Systems Inc, USA) as per manufacturer’s protocol. Extracted viral DNA thus obtained, was subjected to real
time PCR amplification using the real time Cobas® TaqMan® HBV test kit (Roche Molecular System, USA) run on Cobas® TaqMan® 48 Analyzer (Roche Diagnostics, USA) as per manufacturer’s instructions. Finally, quantitation of amplified HBV DNA was performed by using Amplilink software (Roche Diagnostics, USA) and expressed as respective viral load.

Isolation of total RNA from PBMCs

From ethylene diamine tetraacetic acid (EDTA) containing whole blood (~4 ml), PBMCs were isolated by density gradient centrifugation using histopaque-1077 (Sigma chemicals, USA) as per suggested procedure. Isolated PBMCs were lysed and total RNA extracted by phase separation using Trizol (Life Technologies, USA). The extracted RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water.

Preparation of complementary DNA (cDNA)

Extracted RNA was reverse transcribed using M-MuLV reverse transcriptase (Fermantas Life Sciences, Germany) and random hexamers to generate cDNA. To rule out the possibility of DNA contamination, 1µg of total RNA was subjected to 1 U of deoxyribonuclease (DNase I amplification grade, Gibco- BRL, USA) treatment. Additionally, control reactions devoid of reverse transcriptase enzyme were employed to negate the possibility of presence of traces of DNA in the reaction. The reverse transcription reaction was carried out (60 min at 37°C) with 200 U of M-MuLV reverse transcriptase (Fermantas Life Sciences, Germany) in 20 µL volume of 5 × RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂) supplemented with 5 mM dithiothreitol (DTT), 0.5 mM deoxynucleoside triphosphates (dNTPs, Fermantas Life Sciences, Germany), 25 U ribonuclease inhibitor (Promega Corporation, Madison, WI, USA) and 200 ng random hexamers (Fermantas Life Sciences, Germany) in the reaction mixture.

Polymerase chain reaction (PCR) amplification of Atg5 gene

PCR amplification was performed using an aliquot of 5 µL (0.3 µg) of the cDNA as template. The reaction was carried out in 50 µL of 10 × buffer solution (100 mM Tris- HCl pH 9.3, 500 mM KCl, 1% Triton X-100) containing 0.08 mM dNTPs, forward and reverse primers (100 pM each), 1.5 mM MgCl₂ and 2U of Taq DNA polymerase (Fermantas Life Sciences, Germany). Subsequent to preliminary denaturation at 94°C for 5min, Atg5 cDNA were amplified by 30 cycles (94°C for 15sec, 55°C for 30sec & 72°C for 45sec per step) followed by a final extension step at 72°C for 5min. The up and downstream primers employed were (5´-3´) d (AGC AAC TCT GGA TGG GAT TG) and d (CAC TGC AGA GGT GTT TCC AA) respectively (Kim et al., 2011) which capitulated 316 bp amplicon. Amplification of beta actin as housekeeping gene was performed by 30 cycles(94°C, 55°C & 72°C; 1 min per step) using forward and reverse primers (5´-3´) d (TCT ACA ATG AGC TGC GTG TG) and d (GGT GAG GAT CTT CAT GAG GT) generating amplicon of 314bp as mentioned earlier (Shravanthi et al., 2012). Void reactions devoid of cDNA template were executed in all experiments as negative control. Each amplicon (10 µl) was subjected to 2% agarose gel electrophoresis (100V, 45 min) in conjunction with a 100 bp DNA ladder. After staining with ethidium bromide, bands were visualized by UV fluorescence. Upon capturing the gel image by Bio-Capt imazer (Vilber Lourmat, France), the integrated density of respective DNA band was estimated by using the software Image J 1.42 (Broken Symmetry Software, USA) in respect to the known standard marker as a control, with units nanogram per milliliters (ng/ml). The values were further normalized against the housekeeping gene beta actin used as internal control to define the expression of respective genes by the density of the band (Mukherjee et al., 2013).

Polymerase chain reaction amplification of HBV pregenomic RNA (pgRNA)

Amplification of pgRNA was performed using cDNA fragments obtained by reverse transcription of PBMC derived Trizol extracted total RNA as mentioned above. Reaction was carried out for 40 cycles (94°C for 30 s, 58°C for 30 s & 72°C for 30 s per step), subsequent to a preliminary denaturation at 95°C for 5min and afterward a final extension at 72°C for 10 min using forward and reverse primers (5´-3´) d(GCCTTAGAGTCTCCTGAGCA), and d(GAG GGA GGT CTT CTT CTA GG) as stated before (Mukherjee et al., 2013). Finally, the amplicons were explicated by 2% agarose gel
Table 1. Demographic and clinical and virological features of the study subjects

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE</td>
<td>30±6.5</td>
<td>40.97±15.5</td>
</tr>
<tr>
<td>M:F Ratio</td>
<td>24.0</td>
<td>35.5</td>
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<tr>
<td>AST(IU/L)</td>
<td>29.7±5.2</td>
<td>45.8±9.3</td>
</tr>
<tr>
<td>ALT(IU/L)</td>
<td>25.7±7.5</td>
<td>49.8±9.7</td>
</tr>
<tr>
<td>HBV DNA(Log Copies/ml)</td>
<td>-</td>
<td>4.29±0.79</td>
</tr>
<tr>
<td>HBV pgRNA(ng/ml)</td>
<td>-</td>
<td>0.61±0.11</td>
</tr>
</tbody>
</table>

Figure 1. Representative pattern of Atg5 PCR products (316bp) obtained from PBMCs separated by 2% agarose gel. (A) cells from controls and (B) cells from patients; NC=Negative control; L=100bpDNA Ladder

Electrophoresis and staining with ethidium bromide to resolve the desired band size of 364 bp followed by densitometric evaluation. To exclude the possibility of contamination from plasma rather than cells, parallel plasma samples of respective isolated PBMCs were extracted and amplified in identical manner to serve as controls.

Statistical analyses

Using the GraphPad software (GraphPad, San Diego, CA, USA), descriptive statistics (mean, median, standard deviations) and Student’s t test were used to analyze the results. Box-plots and Inter Quartile Ranges (IQR) were used to express the results. A value of p<0.05 was deemed statistically significant.

RESULTS AND DISCUSSION

The detailed demographic, biochemical and virological characteristics of 42 patients and 24 controls are shown in Table 1. The patient group comprised of adult subjects (Mean age ±SD=40.9±15.5 years) majority of whom were male. Control subjects were all males having a mean ±SD age of 30.0±6.5 years.

In contrast to controls, the expression of Atg5 gene was augmented as revealed by greater intensity of PCR bands in HBV infected PBMCs of patients (Figure1 A,B). Comparative densitometric analysis of band intensities upon normalization of values, against the housekeeping gene beta actin, further revealed the upregulated expression of Atg5 gene in HBV infected PBMCs. It was evident that Atg5 gene expression was significantly bumped up (p=0.0001) in HBV infected cells having median value of 155.7 ng/ml (mean ±SD=147.3±45.1, IQR=78.5) against the median value of 94.9 ng/ml (mean ±SD=87.2±34.7, IQR=54.7) in non infected cells obtained from healthy controls (Figure 2).

Autophagy, is a vital metabolic process that can purge superfluous, dented and exhausted cellular components as well
as intercede innate and adaptive immunity against intracellular microbial pathogens. However, some viruses can exploit the host cellular autophagic machinery to modulate their replication in host cells as an advantageous survival strategy. HBV is known to control and engage autophagy for its advantage to uphold replication. It has been shown that HBV can enhance the autophagic process in hepatoma cells without promoting the degradation by lysosomes where HBV small surface proteins are required to induce autophagy (Li et al., 2011). Experimental findings also illustrated that HBV transfected into the hepatoma cells tempted an early autophagic response, without increasing the autophagic protein degradation, which enhanced viral replication through the binding of HBV X protein (HBx) to PI3KC3(Sir et al., 2010). In addition, Atg5–Atg12 conjugate has been shown to interact directly with the intermediary protein signaling 1(IPS-1) and retinoic acid inducible gene 1(RIG-I) through the Caspase activation and recruitment domains (CARDs) that results in the inhibition of type I IFN production and permits viral replication within the cells (Jounai et al., 2007).

However, due to dearth of good animal models to simulate HBV infection and paucity of human liver tissue, informations related to autophagic response in HBV infection so far restricted to the studies involving cell culture and transgenic mice. In this study, utilizing the HBV infected human PBMCs as a model, we demonstrated for the first time that HBV induce autophagy in infected human lymphocytes acquired from HBV patients, which is in agreement with the previous experimental reports (Donna et al., 2010; Li et al., 2011). Detection of HBV pgRNA in PBMCs as viral replication intermediate, further inveterated the PBMCs as a replication site of HBV as observed earlier (Mukherjee et al., 2013). Our data showed that Atg5 gene, a gene critical for the initiation of autophagy was significantly upregulated in PBMCs obtained from HBV infected subjects corroborating the earlier observations in Atg5 knockout mouse liver (Tian et al., 2011). Simultaneous detection of HBV pgRNA in samples where Atg5 gene expression was enhanced, further point to the specific role of replicating HBV in up regulation of Atg5 suggestive of induction of host’s autophagic system. This appears to be advantageous for the virus by taking refuge within the autophagic vesicles there by preventing its components, assembly of particles and encapsidation procedures from the onslaught of host’s anti viral defence mechanisms.

In conclusion, our study showed that autophagy is required for HBV DNA replication. Augmented expression of Atg5 gene in PBMCs of HBV patients is evocative that HBV can induce autophagy in infected human cell as a part of survival.
strategy of the virus. This raises the possibility that prolonged induction of autophagy in patients may play an important role in HBV pathogenesis, particularly in establishing a chronic infection in the liver as well as heaves the leeway of targeting this pathway for the treatment of HBV-infected patients.

REFERENCES


