Original Article

Plasma Myeloperoxidase Activity and Apolipoprotein A-1 Expression in Chronic Hepatitis B Patients

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Abstract

Background: Hepatitis B virus initiates a complicated cascade process leading to chronic hepatitis B, cirrhosis, and hepatocellular carcinoma. In inflammatory situations, myeloperoxidase is released in plasma and binds to apolipoprotein A-1 in high-density lipoproteins. This study aims to evaluate the level of plasma myeloperoxidase as well as the pattern of plasma proteins in patients with chronic hepatitis B.

Methods: Included in this study were 30 male subjects: 19 chronic hepatitis B patients, 6 HBV-related cirrhotic patients, and 5 healthy controls. Plasma myeloperoxidase was measured using enzyme-linked immunosorbent assay. Proteomic analysis of plasma proteins was performed by two-dimensional gel electrophoresis (2-DE) and mass spectrometry. One way ANOVA was used for data analysis.

Results: Mean plasma myeloperoxidase levels were higher in patients with liver cirrhosis (65.5±12.5; *P*=0.007) and chronic hepatitis B (53.7±10.6; *P*=0.18) when compared with healthy subjects (45±7.6). Moreover, a positive correlation was found between plasma myeloperoxidase levels and hepatic fibrosis stage (r=0.53, *P*=0.002; r=0.63, *P*=0.000). Proteomic analysis showed an altered plasma protein pattern in progressive hepatitis B and down-regulation of the major apolipoprotein A-1 along with the appearance of a variety of spots noted to be apolipoprotein A-1isoforms with different molecular masses.

Conclusion: In this study, progressive liver injury due to HBV infection correlated with higher plasma myeloperoxidase and an altered plasma apolipoprotein A-1pattern.

Keywords: apolipoprotein A-1, chronic hepatitis B, myeloperoxidase, two-dimensional gel electrophoresis

Introduction

Hepatitis B virus (HBV) is not cytopathic for the infected hepatocyte, however, extensive evidence points out the importance of an inflammatory contribution of HBV in liver fibrosis among chronic hepatitis B (CHB) patients.[1,2] Liver fibrosis does not occur at the same rate in all individuals, and in some people with CHB, fibrosis remains stable or may even regress over time. This rate may vary widely depending on host factors such as age and gender.[3] It has been demonstrated that HBV antigens can impair host cell lipid metabolism and induce oxidative stress during inflammatory processes that are responsible for the increase of protein oxidation and death of virally infected cells.[4,5] This is supported by the observation of a higher production of reactive oxygen species and release of enzymes such as myeloperoxidase (MPO) during the host immune response that may result in the activation of hepatic stellate cells as a central event in the development of hepatic fibrosis.[6] MPO is a neutrophil and monocytic enzyme that enhances reactivity of hydrogen peroxide through generation of free radicals and reactive nitrogen species. It promotes tissue damages in hepatitis by releasing inflammatory oxidants and cytokines that activate the bystander hepatocyte host immune response.[6,7]

Apolipoprotein A1 (Apo A-1), as a predominant protein component of high-density lipoprotein (HDL), is secreted by hepatocytes and has important anti-inflammatory properties.[8,9] It is well established that viral infection and inflammation are associated with reduced serum HDL and Apo A-1 levels.[5,10] Experiments in two hepatoma cell lines have revealed an inverse correlation between HBV and cellular levels of Apo A-1 and CIII mRNAs.[11] Other studies have shown that the level of circulating Apo A-I correlates with CHB and CHB-related HCC.[11,12] A low level of this protein implies severe liver cell injury. Recent *in vivo* studies have indicated that MPO binds to HDL for oxidative modification of Apo A-1, resulting in the loss of both cholesterol efflux and lecithin cholesterol acyl transferase activating activities, thus generating a dysfunctional HDL particle.[13] It is hypothesized that HBV influences the function of male and female host cells in different ways.[9,14] Therefore, in this study we enrolled male subjects who were either healthy, diagnosed with CHB or HBV-related cirrhosis to resolve and compare the protein patterns in plasma samples by two-dimensional electrophoresis (2-DE). Furthermore, the expression level of plasma MPO in these subjects was evaluated by an enzyme-linked immunosorbent assay (ELISA) system.

Materials and Methods

Patients and sampling

Totally, of the 30 subjects enrolled for analysis in this study, 5 were healthy subjects and 25 were HBeAg negative, which includ-

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Table 1. Demographic and biochemical characteristics of subjects.

Group	Healthy controls (*n*=5) Mean±SD	CHB (*n*=19) Mean±SD	Cirrhotic (*n*=6) Mean±SD	*P*-value
Age (years)	43.0±7.8	42.5±6.1	47.6±7.1	0.28
Log viral load	—	3.6±1	4.7±1.181	0.041
Stage of hepatic fibrosis				
≤2 n (%)	—	18 (100)	0 (0)	0.001
≥3 n (%)		1 (14.3)	6 (85.7)	
Myeloperoxidase (ng/mL)	45±7.6	53.7±10.5	65.5±12.6	0.012

ed 19 CHB and 6 cirrhotic individuals. To avoid the confounder factor of sex, the study was restricted to males. Chronic viral persistence in patients with hepatitis B infection is defined by persistence of HBsAg positive for more than six months and detectable HBV DNA levels. The presence of chronic HBV and cirrhosis was confirmed by liver biopsies. Stage of fibrosis and histological activity was assessed by Ishak's scoring system[15] in which fibrosis stages S1 to S3 are considered mild to moderate liver disease in the CHB group and extensive fibrosis (S4 to S6) for the cirrhosis group. None of the subjects had de-compensated liver cirrhosis. Detailed information on subjects enrolled in the proteomics analyses are shown in Table 1. Plasma and serum samples were collected and kept frozen at -70°C.

This study was approved by the Institutional Review Board of Tehran University of Medical Sciences.

Sample preparation and two-dimensional electrophoresis (2-DE)

In order to increase plasma proteome resolution, albumin depletion was carried out with the use of an ammonium sulfate precipitation method in accordance with Jiang et al.[16] To 15 µL of plasma, 84 µL of saturated ammonium sulfate was added and the precipitation product diluted in 100 µL of 10 mM tris (pH 4.8). The mixture was gently mixed for 10 min, incubated for 2 hr at 4°C and centrifuged at 13000 g for 20 min. The supernatant was transferred into the second tube and proteins were precipitated by adding cold acetone. Subsequently, 500 µL of 80% ice-cold acetone was added to the pellet for washing the precipitated sample. Samples were washed twice with 80% ice-cold acetone and 70% cold ethanol, respectively. The air-dried pellet recovered from the precipitation steps was dissolved in rehydration buffer [7 M urea, 2 M thiourea, 2% CHAPS (v/v), 2% carrier ampholyte (CA) pH 3 – 10 (v/v) and 100 mM dithiotreitol (DTT)] and the protein concentration of each sample was determined according to Bradford[17] using BSA as a standard curve. A volume of each sample containing 400 µg of the total protein was applied to an immobilized pH gradient (IPG) strip (17 cm, pH 4 – 7; Bio-Rad) by in-gel rehydration with the passive rehydration method. After 14 hr of rehydration, strips were transferred to an IEF Cell (Bio-Rad) and proteins were focused in the first electrophoretic dimension for 50000 Vh. For running the second dimension, IPG strips were incubated in the equilibration solution [6 M urea, 50 mM tris-HCl (pH 6.8), 30% (v/v) glycerol, 3% (w/v) SDS, 0.002% (w/v) bromophenol blue] for 30 min. Equilibrated strips were then placed on SDS-polyacrylamide gels (16 cm × 20 cm), 12% acrylamide, fixed in place with 0.5% (w/v) agarose and electrophoresis was carried out with a 30 mA constant current per gel.

Subsequently gels were fixed and stained overnight with Coomassie Brilliant Blue G-250 staining solution for three days.[18]

Image analysis and spot selection

For capturing images of the stained gels, we used a Bio-Rad GS-800 scanner. Analysis of the captured images was carried out using the ImageMaster 2D Platinum 6.0 software that allows spot detection. The gel image showing the highest number of spots and the best protein pattern was chosen as the reference template, and spots in the standard gel were then matched across all gels. Each gel was analyzed for novel spot detection and background subtraction. The quantitative difference in percent volume of spots more than 1.5 fold was considered as differential expression variation and selected for analysis by mass spectrometry.

Protein identification

Spots were manually cut from 2-DE gels, placed in 96 well v-shape polypropylene plates and completely dried. The Ettan Spot Handling Workstation (GE Healthcare, UK) was used for automatic in gel digestion of samples. Dried samples were analyzed using a MALDI-TOF/TOF MS (4700 Proteomics Analyzers, Applied Biosystems, UK), performing MS analysis and subsequent MS/MS analysis on as many as ten precursor peptides. Protein identification was performed by searching the NCBI non-redundant protein database provided by the MASCOT search engine (Matrix Science, UK).

Clinical and laboratory assessments

Demographic data including fibrosis stage, HBV DNA level and serological markers were obtained from the patients' database. Plasma MPO was quantified with a commercial sandwich ELISA kit (BIOXYTECH MPO-EIA, Portland, OR, USA). The results were given in ng/mL.

Statistical analysis

Data were expressed as mean values±standard deviation (SD). The one-sample Kolmogorov-Smirnov test was used to check normality assumption of age and MOP variables. One-way ANOVA compared mean MPO and age among the three groups. The Dunnet test was used for post *hoc* analysis and Chi-square test was used to compare the liver fibrosis grade among the three groups. Association between liver fibrosis grade and MPO variables was shown using Spearman correlation. Data was analyzed with SPSS for Windows, Version 18. Values of $P<0.05$ were considered significant.

Results

Clinical findings

Table 1 shows demographic and biochemical characteristics of the subjects. There was a significant difference in plasma

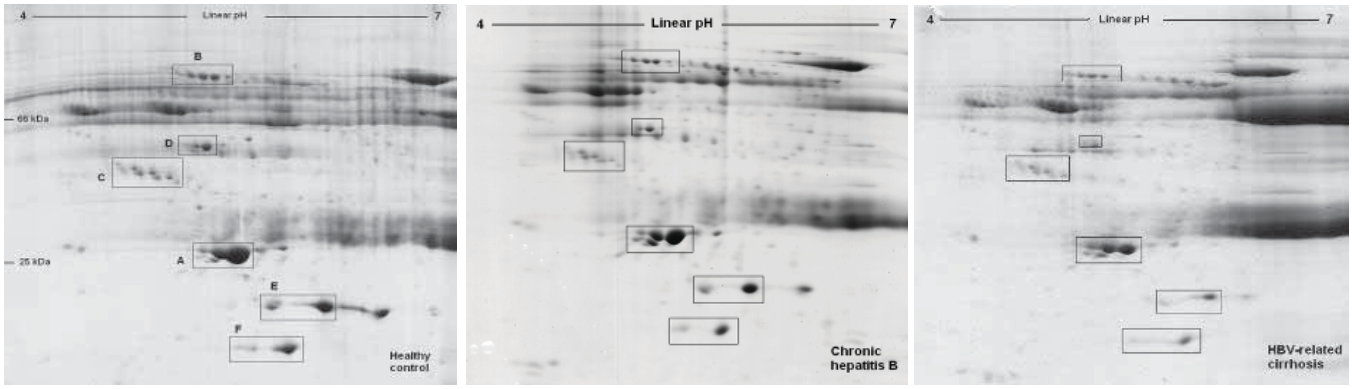

Figure 1. Three representative images of two-dimensional gel electrophoresis obtained from plasma samples of healthy controls, chronic hepatitis B patients, and those with HBV-related cirrhosis. Boxes indicate gel regions that are enlarged in Figure 2.

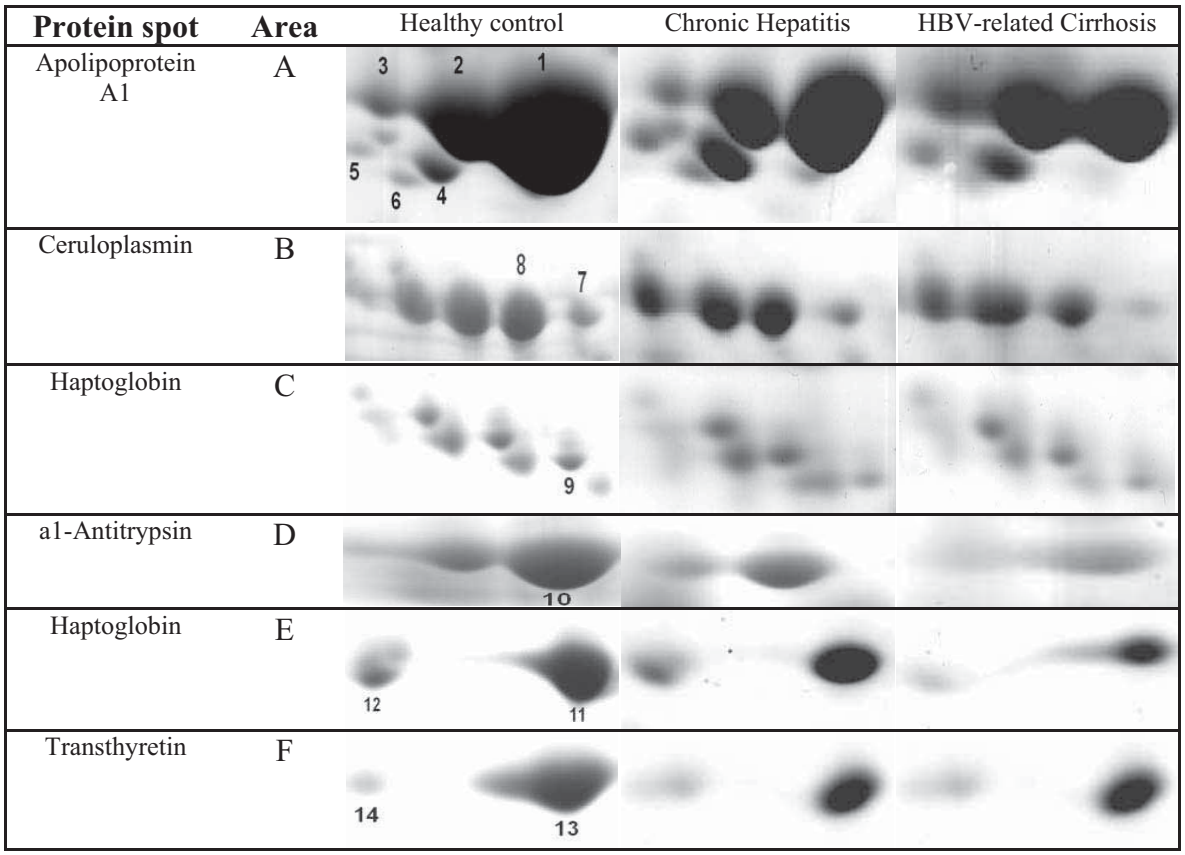

Figure 2. Close-up views of selected gel regions determined by two-dimensional gel electrophoresis that show the locations of protein spots with remarkable changes in their expressions between healthy controls, patients with chronic hepatitis B, and those with HBV-related cirrhosis.

MPO levels (P=0.012) between HBV-related cirrhosis patients (65.5±12.6 ng/mL), CHB patients (53.7±10.6 ng/mL) and healthy controls (45±7.8 ng/mL). Post *hoc* analysis (Dunnet test) showed the mean MPO of patients with cirrhosis was significantly different from normal subjects (mean=20.5, P=0.007), whereas the mean MPO in CHB patients was not significantly different from normal subjects (mean=8.7, P=0.18). There was a positive correlation between plasma MPO and liver fibrosis (r=0.62, P=0.000).The mean deference in MPO of patients with cirrhosis and CHB was 11.7 (95% CI: -3.5 – 21.1; P=0.063). The mean difference in viral load between CHB and cirrhosis patients was significant (P=0.041).

Plasma protein expression patterns

The protein expression profiles of both groups of HBV infected plasma as well as control plasma samples were examined by 2-DE. Figure 1 presents three representative gel images of the healthy reference plasma, HBV infected CHB, and HBV-related cirrhosis. Boxes A to F specify gel regions magnified in Figure 2. Approximately 400 protein spots in a combined reference image were detected, in which 21 spots displayed marked alterations in the level of expression between CHB and cirrhosis when compared with healthy controls. Mass spectrometry results from 16 spots are summarized in Table 2.

Figure 1 illustrates sections of 2-DE images showing differen-

Table 2. Plasma proteins identified by mass spectrometry.

	Protein name	Accession number	Protein MW	Protein PI	Biological function
1	Apolipoprotein A-1	gi|90108664	28061.5	5.27	Carrier (cholesterol)
2	Haptoglobin 2 chain	gi|296653	42126	6.25	Hemoglobin scavenger
3	Apolipoprotein A-1	gi|90108664	28061.5	5.27	Carrier (cholesterol)
4	Alpha1-Antitrypsin	gi|226192647	45669.4	5.81	Protease inhibitor
5	Alpha1-Antitrypsin	gi|226192647	45669.4	5.81	Protease inhibitor
6	Apolipoprotein A-1	gi|90108664	28061.5	5.27	Carrier (cholesterol)
7	Apolipoprotein A-1	gi|2914175	23389.1	5.55	Carrier (cholesterol)
8	Apolipoprotein A-1	gi|90108664	28061.5	5.27	Carrier (cholesterol)
9	Apolipoprotein A-1	gi|90108664	28061.5	5.27	Carrier (cholesterol)
10	Haptoglobin	gi|1212947	38940.5	6.27	Hemoglobin scavenger
11	Haptoglobin	gi|61368204	39097.6	6.13	Hemoglobin scavenger
12	Ceruloplasmin	gi|1620909	116196.7	5.48	Carrier
13	Ceruloplasmin	gi|157831597	120807.7	5.41	Carrier
14	Haptoglobin Hp2	gi|223976	42344.1	6.23	Hemoglobin scavenger
15	Transthyretin	gi|212374952	13797.9	5.35	Carrier
16	Transthyretin	gi|224510585	12995.5	5.33	Carrier

tially expressed spots in the two groups of patients and healthy controls. Among the identified peptides, significant change was observed in the expression profile of haptoglobin, α1-antitrypsin, ceruloplasmin, transthyretin, and the main isoform of Apo A-1 towards cirrhosis. Protein alteration in spots 9, 11, and 12 were decreased in cirrhotic patients. These spots correlate to haptoglobin a2 and B chains that present their own specific train of peptides in a 2-DE gel profile. The expression level of transthyretin (spot 13), ceruloplasmin (spots 7 and 8), and α1-antitrypsin (spot 10) were apparently decreased in both CHB and HBV-related cirrhosis patients. A comparison of 2-DE images of healthy controls, CHB and HBV-related cirrhosis patients revealed an altered pattern of the Apo A-1 in HBV infection. According to the electrophoretic migration and peptide mass spectra, six protein spots with altered expression levels were identified as Apo A-1. Moreover, Figure 1 displays observed changes in Apo A-1 spots corresponding to spots 1 to 6. The locations of spots 1 to 6 of Apo-A1 are consistent with the migration of this protein in healthy subjects, but the pattern of Apo A-1 expression was altered. The net production of Apo A-1 is down regulated in HBV-related cirrhosis. Spot 1 expression showed a significant reduction trend toward cirrhosis patients. Spot 2 was up regulated and spots 3-6 significantly increased in CHB.

Discussion

In the present study, MPO plasma levels were significantly higher in HBV-related cirrhosis and CHB patients than healthy subjects. In addition, we compared plasma protein profiles from healthy controls with CHB and HBV-related cirrhosis patients to find differentially expressed proteins that could be related to disease progression. Although the 2-DE patterns of haptoglobin, α1-antitrypsin, transthyretin, and ceruloplasmin showed altered expression levels in our patients, special attention was paid to Apo A-1 because of the altered migration pattern as well as down regulated expression of the main isoform of this protein. Earlier studies have demonstrated that MPO selectively binds to Apo A-1 in HDL.[13] CHB leads to the gradual destruction of liver tissues and consequent complications that eventually lead to hepatic cirrhosis, which is the major risk factor for the development of HCC.[3] Abnormal liver functions are the basis for altered liver protein synthesis, initiators for pathological features, as well as the development and prognosis of liver injury.[5,6,19] There are contradictory results about the levels of plasma proteins in various liver diseases. Earlier studies reported that haptoglobin, α1-antitrypsin, and APO A-1 decreased in chronic viral hepatitis while other studies showed different results. These conflicting results may be a result of different measurement methods, sample sources, and disease stages.[20,21]

MPO can be used as a marker of inflammation that is caused either by diseases such as asthma, or by environmental irritants. However, only few studies evaluated MPO levels in CHB. Nakamuta et al.[22] have reported increased MPO levels in cirrhotic patients and suggested that high MPO levels might play an essential role in the defense mechanism against invading pathogens.

This study has proven an altered pattern of Apo A-1 in which the average intensity of the major isoform of Apo A-1 decreases with progressive liver disease, whereas fragmentation increases. This observation is in accordance with former studies that have demonstrated an association of chronic hepatitis with the appearance of new Apo A-1 isoforms.[23] Moreover, a different pattern of Apo A-1 isoform expression in male HBVTg mouse liver and CHB patient serum has been reported.[24] It has been reported that MPO/Apo A-1 interactions might play an important role in the development of dysfunctional HDL and the atherosclerotic phenotype.[13,25] Therefore, re-arrangement of the Apo A-1 in MPO-catalyzed oxidation may contribute to altered normal Apo A-1 functions.[24,25] These results are consistent with the concept that NO and MPO contribute to lipid peroxidation in liver tissue caused by free oxygen radicals and is considered to be the main cause of hepatocyte injury.

In summary, this study demonstrated that plasma levels of MPO increased with progressive liver disease. On the other hand, the changing protein profile pattern suggested a useful method for assessing HBV infection.

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