Evaluation of Serum Hyaluronic Acid Level and Hyaluronidase Activity in Acute and Chronic Hepatitis C

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Following major tissue injury, hyaluronic acid production increases as a rapid response survival mechanism. Increased hyaluronic acid production and turnover are often associated with increased hyaluronidase activity, the enzyme that degrades hyaluronic acid. We investigated whether hyaluronic acid and hyaluronidase can be used as non-invasive markers of acute disease activity in hepatitis C by studying 26 patients with acute hepatitis C, 89 with chronic hepatitis C and 32 healthy controls. Chronic hepatitis C subjects were classified into five subgroups according to the stage of liver fibrosis. Serum aspartate aminotransferase and alanine aminotransferase activities and hyaluronic acid levels were increased in hepatitis C patients compared with the controls. Serum hyaluronic acid elevation correlated with disease progression. Serum hyaluronidase activities were also increased in patients compared with the controls, but decreased with disease progression. We conclude that both hyaluronidase and hyaluronic acid may be useful as early non-invasive serum indicators of disease activity in acute hepatitis C.

KEY WORDS: HEPATITIS C; FIBROSIS; CIRRHOSIS; HYALURONIC ACID; HYALURONIDASE

Introduction

Viral liver disease remains a common and challenging problem for clinicians. Hepatitis C virus (HCV) is one of the major causes of acute hepatitis and chronic liver disease, which slowly progresses to fibrosis and cirrhosis.¹,² Liver fibrosis, a final common pathway in the development of chronic liver disease, is characterized by the elevation of both collagenous and non-collagenous extracellular matrix in the liver parenchyma. Excessive matrix deposition is triggered by a disruption in the balance between the synthesis and degradation of extracellular matrix.³,⁴

Hyaluronic acid, also known as hyaluronan, is a straight-chain glycosaminoglycan polymer of the extracellular matrix, where it either aggregates with proteoglycans or it binds to cell surfaces by specific receptors.⁵,⁶ Normally, low concentrations of hyaluronic acid circulate in the blood,
However elevated serum hyaluronic acid levels occur very early after major tissue injury as part of a rapid response survival mechanism. Liver damage is an example of a major tissue injury.5–10

Hyaluronidase, the catabolic enzyme involved in the degradation of hyaluronic acid, is now recognized to be a member of an enzyme family with high sequence homology. Six hyaluronidase-like enzymes have been found in the human genome,11 but hyaluronidase-1 is unique because it is the only one that is found in the mammalian circulation.12,13 Increased hyaluronic acid production and turnover are often associated with increased hyaluronidase levels.

Recent studies have shown that hyaluronic acid production exceeds that of other components of the extracellular matrix in liver fibrosis and, therefore, serum hyaluronic acid levels have been used clinically to assess liver function.14 Furthermore, increased serum hyaluronidase activity also precedes the appearance of hyaluronic acid, so measuring the activity of this enzyme in serum may also provide a very early indicator of liver damage. We investigated whether hyaluronic acid and hyaluronidase levels could: (i) be used to monitor acute disease activity; and (ii) detect early fibrotic processes in patients with acute and chronic hepatitis C.

Patients and methods

PATIENT POPULATION

The study population comprised patients with acute and chronic hepatitis C, as confirmed by liver biopsy, and healthy controls who attended the hepatology clinic of gastroenterology (Cerrahpasa Medical Faculty, Istanbul University). Liver fibrosis was staged on a scale of 0 – 4 using the Metavir scoring system: F0 – F1, no/mild liver fibrosis; and F2 – F4, moderate to severe fibrosis.15 Serum from all patients was tested for antibodies to HCV with the Ortho third-generation HCV enzyme-linked immunoassay (ELISA) Test System (Ortho Diagnostic Systems, Raritan, New Jersey, USA) and positivity was confirmed by reverse transcription-polymerase chain reaction (in-house HCV RT-PCR assay). Non-fasting serum samples were collected from patients at or near the time of their liver biopsy and stored at −70 °C until analysis. Serum samples from healthy volunteers served as controls. The study protocol was approved by the Ethics Review Committee of Istanbul University Cerrahpasa Medical Faculty and all patients provided informed consent.

MATERIALS

Human umbilical cord hyaluronan and all other reagents were obtained from Sigma Chemical Co. (St Louis, Missouri, USA). A commercially available micro-ELISA test kit (Hyaluronic Acid Test Kit, Corgenix, Westminster, Colorado, USA) was used to measure serum hyaluronic acid levels.

BIOCHEMICAL ANALYSES

Hyaluronidase assay

Serum (10 μl) was incubated with 250 μl buffered substrate solution (0.10 mol/l sodium formate, pH 3.9 containing 0.1 mol/l sodium chloride, 250 mg/l hyaluronan and 1.5 mmol/l saccharic acid 1,4-lactone) for 4 h at 37 °C. The enzyme reaction was specifically terminated by addition of 50 μl 0.8 mol/l potassium tetraborate at pH 9.1 to each sample. The tubes were heated for 3 min in a boiling water bath and cooled in tap water. p-Dimethylaminobenzaldehyde reagent (1.5 ml), prepared as described by Reissig et al.,16 was added to each sample, and these were then vortexed, heated at 37 °C for 20 min, briefly centrifuged and read
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using a colorimeter at 585 nm. Consequently, the amount of reaction product (reducing N-acetylglucosamine termini) was determined. Negative controls for the reaction consisted of tubes in which the buffered substrate was incubated for 4 h at 37 °C in the absence of serum which, subsequently, received potassium tetraborate, then serum and were then treated as described above. A standard curve was formed by using known concentrations of N-acetylglucosamine. For this method, 1 unit of hyaluronidase activity was defined as the production of 1 µmol/min of reaction product (reducing terminal N-acetylglucosamine) at 37 °C.17

Hyaluronic acid assay
Serum hyaluronic acid was measured in an enzyme-linked sandwich assay (Corgenix hyaluronic acid test kit) using hyaluronic acid binding protein, following the manufacturer’s instructions and quantified in the concentration range of 10 – 800 ng/ml.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assay
The ALT and AST activities in serum were measured by automated systems using the Roche Hitachi 912 (Roche Diagnostics, Istanbul, Turkey).

STATISTICAL ANALYSES
The results were statistically evaluated using Student’s t-test and one-way analysis of variance (Tukey HSD, Dunnett). Statistical analyses of the parameters were performed by using the SPSS® statistical program version 12.0 (SPSS Inc., Chicago, Illinois, USA). A value of P < 0.05 was considered statistically significant for all analyses.

Results
This study investigated a total of 147 subjects (98 men, 49 women). A total of 26 patients had acute hepatitis C, 89 patients had chronic hepatitis C as confirmed by liver biopsy, and 32 patients were healthy controls. Chronic hepatitis C subjects were classified into five subgroups according to the stage of liver fibrosis (F0 – F4).15 The patient characteristics and the laboratory findings of the three groups are detailed in Table 1.

Serum AST and ALT activities were significantly higher in patients with either acute or chronic hepatitis C when compared with the control subjects (P < 0.001) (Table 1). In addition, AST and ALT were significantly increased in acute hepatitis C patients compared with chronic hepatitis C patients (P < 0.001).

Statistically significant differences in serum hyaluronic acid levels were observed between the groups (Table 2). Patients with acute and chronic hepatitis C had significantly higher levels than controls (P < 0.001). In Fig. 1A serum hyaluronic acid levels are presented as box and whisker plots for patients grouped according to the stage of liver fibrosis. Serum hyaluronic acid levels for these two subgroups were as follows: 149 ± 9.16 ng/ml for stages F0 – F1 and 238 ± 9.04 ng/ml for stages F2 – F4. The difference in the hyaluronic acid levels was statistically significant between these two subgroups (P < 0.01).

Serum hyaluronidase activities were increased in acute hepatitis C patients when compared with the control subjects, but decreased significantly in chronic hepatitis C patients, particularly in those with disease progression as indicated by fibrosis stages F2 – F4 (P < 0.01) (Table 2). In Fig. 1B, serum hyaluronidase activities are presented as box and whisker plots for patients grouped according to the stage of liver fibrosis. Hyaluronidase activities for these two subgroups were as follows: 2088 ± 340 mU/l for stages F0 – F1, and 1720 ± 305 mU/l for
TABLE 1: Demographic characteristics and laboratory findings for the patients and control subjects who participated in this study (n = 147)

<table>
<thead>
<tr>
<th></th>
<th>Patients with acute hepatitis C</th>
<th>Patients with chronic hepatitis C</th>
<th>Control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>26</td>
<td>89</td>
<td>32</td>
</tr>
<tr>
<td>Age (years)</td>
<td>41.63 ± 13.31</td>
<td>43.60 ± 12.30</td>
<td>33.04 ± 7.39</td>
</tr>
<tr>
<td>Male</td>
<td>17</td>
<td>67</td>
<td>14</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>309.20 ± 48.27***</td>
<td>80.52 ± 43.09***</td>
<td>22.62 ± 8.36</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>347.28 ± 74.25***</td>
<td>90.54 ± 42.25***</td>
<td>20.91 ± 7.45</td>
</tr>
<tr>
<td>Metavir fibrosis stage</td>
<td>15</td>
<td>19 (21%)</td>
<td></td>
</tr>
<tr>
<td>F0</td>
<td></td>
<td>F1 (39%)</td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td></td>
<td>F2 (16%)</td>
<td></td>
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<tr>
<td>F2</td>
<td></td>
<td>F3 (10%)</td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td></td>
<td>F4 (14%)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SD or numbers (percentage) of total subjects in each group. ***P < 0.001 compared with the control group.
AST, aspartate aminotransferase; ALT, alanine aminotransferase.

FIGURE 1: Box and whisker plots for hyaluronic acid levels (A) and hyaluronidase activity (B) in patients with chronic hepatitis C grouped according to the stage of fibrosis. The box represents the 25th and 75th percentiles and the median is shown by a horizontal line. The whiskers represent the upper and lower range of values for each marker. The differences between the F0 – F1 and F2 – F4 groups for each of the two markers were significant (P < 0.01)
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The difference in the hyaluronidase activities was statistically significant between these two subgroups ($P < 0.01$).

Discussion

The present study investigated whether both hyaluronic acid and hyaluronidase can be used to monitor early disease activity with a view to preventing progression to the chronic stage in patients with acute hepatitis C.

Previous studies have suggested that both hyaluronic acid and hyaluronidase appear to be very early serum indicators of disease activity in acute liver injury, occurring almost before serum peaks of aminotransferase activities. Based on this hypothesis, we found that both serum hyaluronic acid levels and hyaluronidase activities were higher in patients with acute hepatitis C compared with control subjects. Serum hyaluronic acid levels were presumably elevated due to the damage incurred by the liver endothelial cells and, following increased hyaluronic acid deposition, hyaluronidase activity was probably elevated due to increased hyaluronic acid turnover.

Under normal conditions, these acute effects are reversible when liver endothelial cells recover. Unfortunately, recovery is rare in typical cases of acute hepatitis C. We were interested, therefore, in identifying those patients with a progressive disease course for whom there still might be an opportunity to offer active disease management before the development of fibrosis. Repeated studies of serum hyaluronic acid concentrations in chronic liver diseases have demonstrated that hyaluronic acid may be a useful indicator of progressive liver damage. Endothelial cells take up hyaluronic acid by specific receptors and it is degraded in the lysosomes by intracellular processes. In fibrosis,
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Decreased activity of hepatic sinusoidal cells and a decreased number of specific endothelial cell receptors probably lead to decreased removal of hyaluronic acid from the blood by the liver. Both increased release of hyaluronic acid from the liver and a decreased removal from the blood are possible causes of elevated serum hyaluronic acid levels.

Similarly, in the later stages, with the onset of chronic hepatitis C, fibrotic processes may cause a reduction in the rate of hyaluronidase synthesis and decreased enzymatic activity with an associated reduction in hyaluronic acid degradation, which may lead to increased levels of circulating hyaluronic acid and decreased enzyme activities in the chronic stage. Indeed, we found a significant reduction in serum hyaluronidase activities in patients with chronic hepatitis C, whereas hyaluronic acid levels were elevated.

Following liver damage, cellular lysosomes are ruptured and enzymes, such as hyaluronidase, are released into the cytoplasm. When the integrity of the lysosomal membrane is compromised, alterations to the optimum pH may trigger a reduction in degradation of the substrate hyaluronic acid. Unless an acid pH similar to that within the lysosomes is maintained, hyaluronidase will remain inactive. This may also reduce serum hyaluronidase activity during progression of chronic hepatitis C.

We also found a significant difference between the stages of liver fibrosis, F0 – F1 and F2 – F4, for both hyaluronic acid and hyaluronidase. Subsequent increases in hyaluronic acid concentrations and decreases in hyaluronidase activities due to the severity of fibrosis may help clinicians evaluate disease progression.

We found elevated serum transaminase activities in acute hepatitis C due to acute liver injury. In the later stages with the progression to chronic hepatitis C, serum transaminase activities were progressively decreased, confirming the long-term disruption of liver function.

Although serum transaminase activities are routinely used to measure liver damage, serum hyaluronic acid concentrations and hyaluronidase activities may also be useful markers for assessing the early stage of acute hepatitis C. At present, there is no other clinical measure that clinicians can use to monitor acute liver injury. During the progression of chronic hepatitis C, alterations in serum hyaluronic acid levels and hyaluronidase activities may also give information about the severity of the liver damage.

We conclude that hyaluronic acid-associated parameters might become the leading non-invasive markers of liver fibrosis in the future, which could be used: (i) to indicate the severity of liver damage; and (ii) to monitor the pathological state of the liver, whilst possibly eliminating the necessity for a liver biopsy.

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Conflicts of interest
No conflicts of interest were declared in relation to this paper.

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