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Serum Hyaluronan: A Marker of Liver Fibrosis in Patients with Chronic Liver Disease

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SUMMARY **The aim of this study was to evaluate the clinical significance of serum hyaluronan (HA) as a marker of liver fibrosis in patients with chronic liver disease. Serum HA was measured by an ELISA-based method in 28 patients with chronic hepatitis (CH), 43 patients with liver cirrhosis (LC), 57 patients with hepatocellular carcinoma (HCC) and 60 healthy controls. Mean serum HA concentration in patients with LC was 1,376.80 ± 2,568.85 ng/ml which was significantly higher than those in patients with CH, HCC and the controls (575.93 ± 732.58, and 426.36 ± 687.33, and 117.86 ± 311.11 ng/ml, respectively). Based on a ROC curve analysis, a cut-off point of 354 ng/ml discriminated between LC and other groups with a sensitivity, specificity and accuracy of 82.4%, 78.2%, and 80.2%, respectively. Mean HA concentrations were correlated with the degree of liver fibrosis, but not the grade of necroinflammatory activity. In patients with LC, the mean serum HA level was significantly increased in the Child C group (3,977.96 ± 4,906.21 ng/ml) in comparison with the Child B and A groups (1,002.63 ± 448.55, and 537.90 ± 424.16 ng/ml, respectively). We conclude that serum HA concentrations reflect the extent of liver fibrosis and severity of cirrhosis. Thus, serum HA can be a diagnostic marker of liver fibrosis and cirrhosis in patients with chronic liver disease.**

Chronic liver disease of any etiology may lead to the development of liver fibrosis and cirrhosis. Liver fibrosis is characterized by increased deposition and altered composition of extracellular matrix (ECM) components in the portal tracts, around central veins or in perisinusoidal spaces.[1] The progresssive accumulation of ECM distorts the liver architecture and consequently compromises hepatocyte function, causing life-threatening complications such as variceal bleeding, ascites and liver failure. Currently, liver biopsy remains the standard method of assessment of liver fibrosis and cirrhosis. However, the use of liver biopsy in clinical practice has several limitations, such as hemorrhage, discomfort, sampling error and the cost of hospitalization. Therefore, several biochemical techniques have been studied as surrogate makers of liver fibrosis, which would obviate or greatly reduce the need of liver biopsy. These biochemical markers include products of collagen synthesis or degradation (e.g. type III procollagen peptide, type IV collagen 7S domain), and serum levels of enzymes involved in matrix turnover (e.g. tissue inhibitor of metalloproteinases). The ECM glycoproteins and proteoglycan/glycosaminoglycan such as fibronectin and hyaluronan have also been examined.[2, 3]

Hyaluronan (hyaluronic acid, HA), ubiquitously distributed in the extracellular spaces, is a linear

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polysaccharide produced from repeating disaccharide units of N-acetylglucosamine and glucuronic acid up to a molecular weight of 10^4-10^7 daltons.[4] Hepatic production of this glycosaminoglycan occurs predominantly in the hepatic stellate cells, the myofibroblast-like cells which are primarily responsible for liver fibrogenesis.[5] HA is degraded locally, but a portion enters the systemic circulation via the lymphatic systems and then is predominantly cleared by receptor-mediated endocytosis in hepatic sinusoidal endothelial cells (SEC), although a small fraction undergoes renal elimination.[6] It has been shown that serum HA levels are low in patients with normal liver tissue, but elevated levels occur in patients with various etiologies of fibrotic liver diseases including chronic viral hepatitis, alcoholic liver disease and others.[2,7] It has also been demonstrated that high serum HA levels are associated with clinical severity and complications of cirrhosis.[8] Indeed, most previous studies were performed in Japan and Western countries where chronic hepatitis C and alcoholic liver disease are common. However data from endemic areas of chronic hepatitis B, such as Southeast Asia, are still lacking.

The aim of this study was, therefore, to evaluate the clinical significance of serum HA as a marker of liver fibrosis in Thai patients with chronic liver disease by demonstrating that its levels were correlated with the degree of fibrosis and the severity of cirrhosis.

MATERIALS AND METHODS

Patients

Twenty-eight patients with chronic hepatitis (CH), 43 patients with liver cirrhosis (LC) and 57 patients with hepatocellular carcinoma (HCC) were randomly selected from a pool of patients with chronic liver disease who were followed up at King Chulalongkorn Memorial Hospital, Bangkok, Thailand, between August 1997 and September 1999. Serum samples were collected from each patient at the time of their clinical evaluation and stored at -70^0C until further tested. Sixty stored sera randomly collected from voluntary blood donors at the National Blood Center, Thai Red Cross, between November and December 1997 served as control samples. The study was approved by the ethic committee, Chulalongkorn University.

The diagnosis of CH was based on a prolonged elevation of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and was confirmed by histological examination. CH was subclassified according to the grade of necroinflammatory activity, which is defined by the combination of the first three categories of the Knodell classification:[9] necroinflammatory scores: ≤ 3 for minimal CH, 4-8 for mild CH, 9-12 for moderate CH, and 13-18 for severe CH. The CH group comprised 26 males and 2 females with a mean age of 40.6 ± 11.5 years. Eight patients had minimal CH (7 were HBsAg positive, 1 was anti-HCV positive), 18 patients had mild CH (13 HBsAg positive, 4 anti-HCV positive, 1 positive for both markers), and 2 patients had moderate CH (1 HBsAg positive, 1 without hepatitis marker). None of the patients had severe CH.

The diagnosis of LC was based on liver biopsy in 7 patients and on a combination of clinical and radiological findings in the remaining 36 patients. In this group, there were 32 males and 11 females with a mean age of 49.9 ± 12.3 years. All patients were classified according to Child criteria: Child A (23 patients, 10 HBsAg positive, 1 anti-HCV positive and 1 positive on both markers), Child B (11 patients, 6 HBsAg positive and 2 anti-HCV positive), and Child C (9 patients, 1 HBsAg positive, 3 anti-HCV positive).

The diagnosis of HCC was based on histopathology and/or a combination of mass lesions in the liver on hepatic imaging as well as on serum alpha-fetoprotein (AFP) levels above 400 ng/ml. There were 51 males and 6 females with a mean age of 53.4 ± 12.2 years in this group. 42 patients were HBsAg positive, 2 anti-HCV positive and 1 had both markers positive.

Laboratory methods

The serum HA level was determined by a competitive inhibition based-ELISA as previously described with modifications.[10] Briefly, serum samples containing unknown amounts of HA, as well as a standard containing known concentrations of a highly purified HA preparation (Healon® in 6% bovine serum albumin) were placed in small polypropylene tubes with appropriate concentrations of biotinylated-HA binding proteins (B-HABP) and incubated at room temperature (25°C) for 1 hour. Aliquots (100 µl) of this reaction mixture were applied to umbilical cord HA coated and BSA blocked microtiter plates and incubated at 25°C for 1 hour. The wells were then washed with phosphate buffered saline solution (0.05% Tween-20) and the

appropriate dilution of anti-biotin peroxidase conjugate (Zymed Lab Inc, San Francisco, CA, USA) was added to each well, incubated at 25°C for 1 hour and washed, after which peroxidase substrate was added. After incubation at 25°C for 20 minutes, the reaction was stopped by the addition of 50 µl 4 M H_2SO_4. The absorbance ratio at 492/690 nm was measured using a Titertek Multiskan M340 microplate reader. The level of HA in the serum samples was determined by their ability to inhibit color development in the assay relative to a standard curve generated from the purified HA preparation.

Statistical analysis

Data were presented as a mean ± standard deviation. Comparisons between groups were analyzed by a two-tail ANOVA analysis. A p-value < 0.05 was considered statistically significant. A receiver-operating characteristic (ROC) curve was constructed to establish the diagnostic cut-off level of serum HA in order to discriminate LC from other groups. Sensitivity, specificity and accuracy were calculated in accordance with standard methods.

RESULTS

Clinical characteristics and mean HA concentrations of patients in each group are shown in Table 1. The mean serum HA concentration in the controls was 117.86 ± 311.11 ng/ml. Compared to the controls, the mean serum HA level was significantly increased in patients with LC (1,376.80 ± 2,568.85 ng/ml) (p < 0.001), but not in patients with CH (575.93 ± 732.58 ng/ml) (p = 0.13) and patients with HCC (426.36 ± 687.33 ng/ml) (p = 0.21). A significant difference of serum HA concentration was also observed between patients with LC and CH (p = 0.01), as well as between patients with LC and HCC (p < 0.001). However, there was no significant difference of mean serum HA levels between patients with CH and HCC (p = 0.63).

In order to discriminate LC from other groups with an optimal accuracy, an analysis of the ROC curve was performed. As shown in Fig. 1, the area under the curve of LC and the other three groups was 0.840 [95% confidence interval (CI) 0.782-0.982]. This result indicated that 84% of randomly selected patients with LC would have a higher HA value than a patient randomly selected from the other groups. Based on the ROC curve analysis, the cut-off point for the serum HA concentration to achieve the highest accuracy for the diagnosis of LC was 354 ng/ml. At this concentration, the sensitivity, specificity and accuracy for differentiating between LC and other groups were 82.4%, 78.2%, and 80.2%, respectively.

Among patients who had liver biopsy, the mean HA concentrations were not correlated with the grade of necroinflammatory activity (Table 2). Conversely, patients who had severe or bridging fibrosis (F3) or cirrhosis (F4) had higher mean HA concentrations than those with less severity (Table 3). In addition, among patients with LC, serum HA levels significantly correlated with the Child classification (Table 4). We found that the mean serum HA concentration was significantly increased in patients with Child C (3,977.96 ± 4,906.21 ng/ml) in comparison to patients with Child B (1,002.63 ± 448.55 ng/ml, p = 0.04) and Child A (537.90 ± 424.16

Table 1 Clinical characteristics and serum HA levels

Group	No.	M/F	Age (years)	TB (mg/dl)	AP (IU/l)	AST (IU/l)	ALT (IU/l)	Alb (g/dl)	Mean HA (ng/ml)	Median HA (Min-Max) (ng/ml)
Control	60	38/22	34.2 ± 10.3	-	-	-	-	-	117.86 ± 311.11	48.55 (1.28-1,455.60)
CH	28	26/2	40.6 ± 11.5	0.7 ± 04	223.4 ±284.8	71.8 ± 49.3	119.3± 103.7	4.6 ± 0.4	575.93 ± 732.58	489.37 (38.98-3,876.25)
LC	43	32/11	49.9 ± 12.3	3.1 ± 3.7	278.9 ±173.3	108.7 ± 96.0	84.8±1 12.0	3.6 ± 0.8	1,376.80 ± 2,568.85	688.71 (124.74-13,440.23)
HCC	57	51/6	53.4 ± 12.2	2.3 ± 2.9	532.3 ±450.3	155.3 ± 120.0	86.8±7 9.2	3.5 ± 0.6	426.36 ± 687.33	244.62 (34.89-4,776.42)

CH, chronic hepatitis: LC, liver cirrhosis: HCC, hepatocellular carcinoma
TB, total bilirubin (normal value 0-1.0 mg/dl): AP, alkaline phosphatase (normal value 98-279 IU/L); AST, aspartate transaminase (normal value 0-38 IU/L); ALT, alanine transaminase (normal value 0-38 IU/L); Alb, albumin (normal value 3.4-5.5 g/dl)

Table 2 Mean serum HA levels in patients with CH and LC according to necroinflammatory activity of the Knodell scoring systems

Necroinflammatory activity (score)	N	Serum HA (ng/ml)
Minimal (≤ 3)	12	542.81 ± 461.49
Mild (4-8)	21	608.38 ± 817.54
Moderate (9-12)	2	427.42 ± 156.13

ng/ml, $p = 0.001$). Likewise, the mean serum HA concentration in patients with Child B was significantly higher than that found in Child A ($p = 0.02$).

DISCUSSION

Liver cirrhosis is caused by progressive fibrosis that ultimately results in nodular regeneration with a loss of function of the hepatocytes. Indeed, the accumulation and alteration of several ECM components, including collagens, complex glycoproteins and proteoglycans/glycosaminoglycans, in the development of liver fibrosis and cirrhosis are dynamic processes.[11] As a result, these ECM components have been used as potential biochemical markers of liver fibrosis and cirrhosis. Among them, serum HA is currently considered as one of the most suitable markers available.[2] In patients with chronic liver diseases of various etiologies, increases in serum HA levels occur together with the development of liver fibrosis, suggesting that progressive liver damage can be identified early by this marker.[12] In chronic hepatitis C patients treated with interferon, serum HA levels decrease significantly in those with improved fibrosis, increase in those with worsening fibrosis, and are unaltered when fibrosis is not modified.[12] Moreover, high serum HA levels are correlated

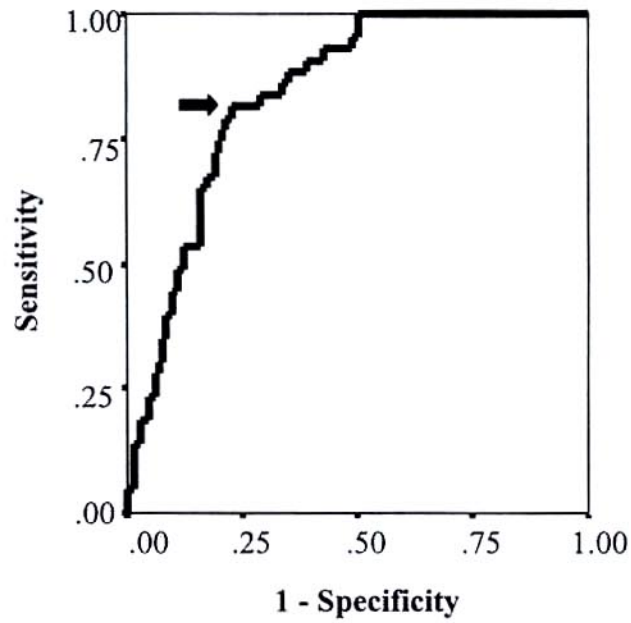

Fig. 1 The ROC curve of serum HA distinguishing between LC and the other three groups (the area under the curve was 0.840). The arrow indicates the cut-off point of 354 ng/ml.

Table 3 Mean serum HA levels in patients with CH and LC according to fibrosis stages of the Knodell scoring systems

Fibrosis stage	N	Serum HA (ng/ml)
Portal fibrosis (F1)	10	220.02 ± 185.91
Septal fibrosis (F2)	7	381.61 ± 168.14
Bridging fibrosis (F3)	11	1,023.15 ± 1,013.83*
Cirrhosis (F4)	7	952.62 ± 344.31*

*$p < 0.05$ versus F1

with the occurrence of severe complications in patients with cirrhosis, and can be used as a good prognostic marker.[8, 13]

In agreement with previous reports, we demonstrated that serum HA concentrations were related to the histological degree of liver fibrosis. Also, serum HA levels were markedly increased in patients with LC, and significantly correlated with the Child classification. However, no relationship between this marker and the histological degree of necroinflammation indicating hepatocellular damage was established. Taken together, our results suggest that serum HA levels may not determine the inflammation of the liver, but rather reflect the extent of liver fibrosis and the severity of cirrhosis. Thus, the measurement of this serum marker may be helpful for diagnosing liver fibrosis and cirrhosis in patients with chronic liver disease. Interestingly, we found that serum HA concentrations were significantly decreased in patients with HCC compared to those with LC, and also there was no difference in the levels of this marker comparing the CH and HCC groups. The explanation of these findings is unclear because HCC usually occurs in the presence of liver cirrhosis. Nonetheless, it has been previously shown that HCC also develops, although less frequently, in a non-cirrhotic liver. Recent data have demonstrated that the prevalence of non-cirrhosis in individuals with HCC and chronic hepatitis B or C is approximately 20-25%.[14] Thus, it could be speculated that a portion of patients with viral hepatitis B- or C-associated HCC in this study might have underlying a non-cirrhotic liver, resulting in relatively low serum HA levels.

Table 4 Mean serum HA levels in patients with LC according to the Child Classification

Child Classification	N	Serum HA (ng/ml)
A	23	537.90 ± 424.16
B	11	1,002.63 ± 448.55*
C	9	3,977.96 ± 4,906.21*#

*$p < 0.05$ versus Child A, # $p < 0.05$ versus Child B

Several mechanisms may contribute to elevated serum HA levels in patients with chronic liver disease. First, hepatic stellate cells are the main cellular source of HA, and it is now recognized that these cells undergo a phenotypic transformation, termed activation, during chronic liver injury.[11] Thus, enhancement of production by activated hepatic stellate cells may be responsible for the increase in serum HA levels as observed in patients with early stages of chronic viral hepatitis.[15] In addition, HA is cleared by receptor-mediated endocytosis in hepatic sinusoidal endothelial cells.[16] Therefore, in advanced disease when hepatic sinusoids capillarization and cirrhosis are established, reduced degradation by sinusoidal endothelial cells may cause increased HA in circulation.[17] Recently, a histochemical study has confirmed that in chronic hepatitis, the production of HA is accelerated in hepatic stellate cells, but degradation of this glycosaminoglycan by sinusoidal endothelial cells continues. On the other hand, in liver cirrhosis, HA production decreases in hepatic stellate cells, and a marked transformation of sinusoidal endothelial cells with sinusoidal capillarization indicates a loss of the ability to degrade HA.[18] Taken together, elevated serum HA is regarded as an indicator of liver fibrosis with activated hepatic stellate cells and dysfunction of sinusoidal endothelial cells. However, it should be noted that the liver is only one of several tissues which contains significant amounts of HA. Thus, elevated serum HA is not specific for liver fibrosis, but can occur in other diseases associated with increased turnover of ECM such as rheumatologic diseases and pulmonary fibrosis.

In conclusion, our data suggest that serum HA concentrations reflect the extent of liver fibrosis and the severity of cirrhosis. Thus, serum HA could be used as a surrogate marker for diagnosing liver fibrosis and cirrhosis in patients with chronic liver disease.

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