

Attenuation of serum laminin concentrations upon treatment of chronic hepatitis

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Abstract

Objectives: The aim of this work was to determine the serum laminin level cutoff point for predicting liver fibrosis highlighting its diagnostic value and determining the effect of treatment on serum laminin concentrations.

Methods: Serum laminin concentrations in chronic hepatitis patients (n=62) and controls (n=20) were compared by ELISA and stages of fibrosis were assessed according to the modified Knodell score system.

Results: Mean serum laminin concentration in patients (91.9 ± 20.9 ng/ml) was greater than controls (46.2 ± 10.2 ng/ml; $p < 0.001$). Serum concentrations of laminin in all stages of hepatic fibrosis were significantly higher than those of healthy controls ($p < 0.05$). A cutoff point of 52ng laminin/ml of serum was obtained for the discrimination of various stages of liver fibrosis showing a good sensitivity (96.8%) and specificity (80%). After 6 months of treatment, a gradual decrease in serum laminin concentrations were observed, however the level was still higher than that of the healthy group ($p < 0.05$).

Conclusions: Our findings suggest that the serum laminin concentration is a useful noninvasive marker of liver fibrosis and shows a strong positive correlation with different stages of the disease.

Key words: chronic hepatitis; hepatic fibrosis; laminin; treatment

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Introduction

Laminin was initially identified by Timpl and Martin in 1979, from a murine fibrosarcoma (1). Laminin is one of the main glycoproteins of the basement membrane and participates in a series of such biological phenomena such as adhesion, migration, cellular differentiation and the maintenance of the cytoskeleton upon its binding to several components of the matrix, such as collagen type IV, heparin sulphate and entacin (2-7).

In the liver, laminin is normally found around the vessels and biliary ducts, where basement membranes are identified. Little or only a slight reaction for antibodies against laminin can be observed in the hepatic sinusoids (8, 9). In this organ, glycoproteins are also involved in intracellular activities, such as the normal differentiation of the

biliary ducts, expression of albumin messenger RNA in hepatocytes, and regeneration with normal lobular organization following partial hepatectomy (10-12). Laminin is thought to be synthesized by hepatocytes and sinusoidal cells (13). Among all cellular types in the sinusoids, special attention should be given to stellate cells or lipocytes, which produce the largest amount of serum laminin. With the development of hepatic cirrhosis, laminin and collagen deposition occurs both along the fibers of septal fibrosis and subendothelial sinusoids or Disse's space. At the latter site, laminin deposition, together with collagen deposition, determine the formation of a true basement membrane along sinusoids. This phenomenon is called capillarization of Disse's space (14).

Increased concentrations of laminin were observed in the more advanced stages of fibrosis in patients with hepatic disease (15-19). Kropf et al have proposed laminin serum concentrations as a sensitive screening test for hepatic fibrotic disease and portal hypertension (18, 19).

An important component of the management of hepatic fibrosis is the clinical assessment of disease severity. Liver histology is frequently considered the gold standard for establishing the severity of hepatic necro inflammation and fibrosis. However, liver biopsy is an invasive procedure that may cause undesirable events, such as pain in 20% to 30% of cases, major complications in 0.5%, and even death. In addition, because of the complications derived from the procedure and frequent poor patient acceptance, the direct costs of such procedures are high (20). Thus, the finding of surrogate markers of liver fibrosis could be relevant to reduce the number of liver biopsies in patients with hepatitis.

The first aim of this study was to determine the serum laminin level cut off point to predict both presence and absence of fibrosis. The second aim was to obtain a relationship between the diagnostic values of serum laminin concentrations for differentiation of various stages of hepatic fibrosis in patients with chronic hepatitis. The final aim was to determine serum laminin changes during treatment of these patients.

Materials and methods

Study population

62 patients (35 men and 27 women, mean age \pm SD: 35.4 \pm 11.3; range: 15-65 years) were enrolled in the study. Among these, 35 patients had hepatitis B virus (HBV), 14 had hepatitis C virus (HCV), and 13 were autoimmune hepatitis (AIH). The subjects were selected from persons who were referred to the Gastroenterology Research Centres in Tabriz and Gonbad, Iran. Patients were included in the study if they were positive for serum hepatitis B surface antigen or C antibodies and had persistently elevated serum aminotransferase concentrations greater than 1.5 times the upper limit of the reference range for at least six months. All patients were diagnosed according to the International Autoimmune Hepatitis Group Report protocol (21).

For assessment of liver fibrosis scores all patients underwent liver biopsy as part of the normal diagnostic procedure and were subclassified according to the score for the histological activity index (HAI). Patients with a history of gastrointestinal bleeding and chronic liver disease (Wilson's disease, hemochromatosis, α 1pha 1-antitrypsin deficiency, biliary disease, hepatocellular carcinoma), active intravenous drug abuse, and liver transplantation were excluded.

Control sera for the determination of laminin were obtained from 20 healthy individuals; 10 women and 10 men, 20-69 years old (mean \pm SD: 42 \pm 14.7 years). These healthy persons had normal serum concentrations of aminotransferases and alkaline phosphatase (ALP) and had no history of gastrointestinal bleeding or chronic liver disease, smoking, alcohol intake, no family history of hepatitis or liver disease, and no active intravenous drug abuse, or liver transplantation. All patients gave written informed consent to use these data for scientific purposes and the study was approved by Tabriz University of Medical Sciences Ethical Committee.

Blood sample collection and analysis

Fasting venous blood (5ml) was collected on the day before the beginning of the treatment and three times at two monthly intervals, i.e. two, four and six months after the beginning of treatment. Serum was separated (2500 g for 5 minutes) within one hour of blood collection. Standard liver function tests (LFT), including aspartate aminotransferase (AST), alanine aminotransferase (ALT), total and direct bilirubin, albumin (Alb), and hepatitis serology were performed on aliquots of each sample at entry and recorded. The rest of the serum samples were stored at -20°C . Serum laminin concentrations were determined in one analytical batch. The controls were dealt with in the same manner, except that the control group provided blood only once at entry. Routine LFT were performed using commercially available kits (Ziestchem, Iran).

Patients treatments were begun if they met the inclusion criteria and they were followed up for at least six months. Treatment of each patient was according to a standard protocol as follows. Hepatitis C patients were treated with Pegylated Interferon + Ribavirin or Interferon + Ribavirin. Hepatitis B patients were treated with Interferon or Adefovir and the AIH patients with Prednisolone and Imuran (22).

Serum laminin concentrations were assayed using a laminin EIA Kit (Takara Bio, code number: MK107) on an ELISA reader (BDLSL, Immunoscan, Switzerland, Lab System). The laminin EIA kit is a solid phase EIA based on a sandwich method that utilizes two mouse monoclonal anti-laminin antibodies to detect laminin by a two-step procedure. One of the monoclonal antibodies is bound to the microtitre plate to create the solid phase. Non-specific binding is blocked using a blocking buffer. Samples and standards are then incubated in the microtitre plate wells. After washing the plate, the second anti-laminin monoclonal antibody that is labeled with peroxidase (POD) is added to the wells and incubated. During these steps, laminin is captured onto the solid support on one side and tagged on the other by POD-anti-laminin. The reaction between

POD and substrate (H_2O_2 and tetremethylbenzidine) results in color development with intensities proportional to the amount of laminin present in the samples and standards. The amount of laminin was determined by measuring the absorbances using an EIA plate reader. A standard curve of 5, 10, 20, 40, 80, 160 and 320 ng/ml laminin was used to convert sample absorbances into ng laminin/ml serum.

Histological assessment of liver damage

All patients underwent a liver biopsy for assessing the presence and severity of liver disease. The biopsy fragments were fixed in a 10% formalin solution for 12 hours and embedded in paraffin. Sections were stained with hematoxylin-eosin, Masson's tri chrome and reticulin stain to establish the histological diagnosis and the extent of the liver lesions.

Specimens were graded and staged according to the modified Knodell scoring system (23, 24). The grading system scores 0-18 and was based on sum of four indices:

1. Periportal or periseptal interface hepatitis (piecemeal necrosis, score 0-4)
2. Confluent necrosis (score 0-6)
3. Focal (spotty) lytic necrosis, apoptosis, and focal inflammation (score 0-4)
3. Portal inflammation (score 0-4)

The fibrosis scores were determined as Stage 0 if there was no fibrosis, Stage 1 if there was fibrous expansion of some portal areas, with or without short fibrous septa, Stage 2 if there was fibrous expansion of most portal areas, with or without short fibrous septa, Stage 3 if there was fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging, Stage 4 if there was fibrous expansion of portal areas with marked bridging [portal to portal (P-P) as well as portal to central (P-C)], Stage 5 if there was marked bridging (P-P and/or P-C) with occasional nodules (incomplete cirrhosis), and Stage 6 if there was probable or definite cirrhosis (23,24).

Statistical analysis

All statistical analyses were done by SPSS version 12.0 for Microsoft Windows (SPSS Inc.) and the data were considered statistically significant at a two-sided $p < 0.05$. Numerical data were expressed as mean \pm SD. According to the Gaussian distribution (1 sample Kolmogorov-Smirnov test), mean of serum laminin concentrations of patients and various chronic hepatitis stages, as well as the control group, were compared using the Mann-Whitney U-test or Student's t test. Spearman's correlation coefficients were calculated to assess the relationship between the histological degree of severe liver fibrosis and the concentrations of serum laminin.

To assess and compare the diagnostic accuracy of laminin for differentiating chronic hepatitis patients with severe liver fibrosis from those without fibrosis, we plotted ROC curves (25) and calculated the areas under the curves (AUC) for comparison. Receiver operating characteristic (ROC) curves were generated by plotting the relationship of the true positivity (sensitivity) and the false positivity (1 - specificity) at various cutoff points of the test. An AUC of 1.0 is characteristic of an ideal test, whereas 0.5 indicates a test of no diagnostic value (26). The diagnostic sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) values were also calculated.

Results

Serological and biochemical profiles of the patients are summarized in Table 1. Hepatitis serology revealed that 56.4% of the patients were suffering from chronic hepatitis B, 22.5% from chronic hepatitis C and 20.9% from autoimmune hepatitis. Histological examination of liver for fibrosis scoring revealed 35% of patients to be suffering from significant fibrosis (stage ≥ 3).

The mean serum laminin concentrations (ng/ml \pm SD) in patients

Table 1. Serological and biochemical profile of patients and control group.

Variables	Patients (n=62)	Control group (n=20)
Male/Female (No)	35 / 27	10 / 10
Age in years (mean ± SD)	35.4 ± 11.3	42 ± 14.7
ALT (mean ± SD) Reference range: < 38 IU/L	132.7 ± 141.7	27.3 ± 6.4
AST (mean ± SD) Reference range: < 42 IU/L	97.0 ± 138.3	28.3 ± 6.5
ALP (mean ± SD) Reference range: women 64-305 IU/L; men 80-306 IU/L	376.4 ± 413.7	130.6 ± 38
Total bilirubin (mean ± SD) Reference range: <20.5 µmol/L	32.5 ± 58.1	-
Direct bilirubin Direct (mean ± SD) Reference range: < 6.8 µmol/L	11.5 ± 23.1	-
Albumin (mean ± SD) Reference range: 35.0-52.0 g/L	41.0 ± 6.0	-
Hemoglobin (mean ± SD) Reference range: women 12.0-16.0 g/L; men 14.0-18.0 g/L	13.1 ± 2.6	-
Hematocrit (mean ± SD) Reference range: women 37.0-47.0%; men 40.0-54.0%	41 ± 9.1	-
Platelets (mean ± SD) Reference range: 160,000-450,000	220,511 ± 132,732	-
Smoking (No, %)	7 (11%)	0 (0%)
Alcohol intake (No, %)	1 (1.6%)	0 (0%)
Family history of hepatitis (No, %)	5 (8%)	0 (0%)
Family history of chronic liver disease (No, %)	1 (1.6%)	0 (0%)
History of drug abuse (No, %)	2 (3.2%)	0 (0%)
Chronic hepatitis B (No, %)	35 (56.4%)	0 (0%)
Chronic hepatitis C (No, %)	14 (22.5%)	0 (0%)
Autoimmune hepatitis (No, %)	13 (20.9%)	0 (0%)
Fibrosis Stage 0 (No, %)	10 (16.1%)	0 (0%)
Fibrosis Stage 1 (No, %)	19 (30.6%)	0 (0%)
Fibrosis Stage 2 (No, %)	11, 17.7%	0, 0%
Fibrosis Stage 3 (No, %)	9, 14.5%	0, 0%
Fibrosis Stage 4 (No, %)	8, 12.9%	0, 0%
Fibrosis Stage 5 (No, %)	4, 6.4%	0, 0%
Fibrosis Stage 6 (No, %)	1, 1.6%	0, 0%

Table 2. Comparison of serum laminin concentrations (ng/ml, mean ± SD) of patients in various stages of liver fibrosis and various stages of sampling vs. healthy controls.

Laminin concentrations	Fibrosis Stage 0	Fibrosis Stage 1	Fibrosis Stage 2	Fibrosis Stage 3	Fibrosis Stage 4	Fibrosis Stage 5
At entry	63.0 ± 12.1†	85.7 ± 6.3‡	94.0 ± 10.2‡	100.6 ± 8.6‡	104.2 ± 20.2‡	130.2 ± 13.7‡
2nd month	55.4 ± 8.5†	79.5 ± 6.6‡	90.9 ± 12.0‡	96.0 ± 7.0‡	100.4 ± 18.4‡	118.2 ± 19.0‡
4th month	49.2 ± 6.0*	75.3 ± 7.0‡	85.7 ± 12.4‡	90.0 ± 5.0‡	93.0 ± 15.2‡	111.7 ± 12.6‡
6th month	46.2 ± 4.5*	75.2 ± 6.0‡	83.8 ± 11.1‡	87.6 ± 5.9‡	88.9 ± 13.0‡	110.2 ± 9.5‡

Results are mean ± SD. Differences: *statistically not significant; †p <0.05; ‡ p <0.001

with HBV, HCV and AIH were 92.0 ± 20.9 (range: 63-149), 92.8 ± 24.2 (range: 43-128), 90.6 ± 18.4 (range: 69-128), respectively. However, the mean serum laminin concentrations (ng/ml \pm SD) in healthy control subjects were statistically significantly lower than the patients serum laminin concentrations (46.2 ± 10.2 ; $p < 0.001$, Figure 1).

In Table 2, mean \pm SD of serum laminin concentrations in various chronic hepatitis stages and various stages of sampling are presented. As shown in this table, differences in serum concentrations of laminin, almost in all stages of hepatic fibrosis as compared with the healthy controls, were not statistically significant ($p < 0.05$). An exception was in 3rd and 4th samples of patients in Stage 0 ($p=0.313$ and 0.985 , respectively). Also in fibrosis Stage 6, which was represented by only one patient, we could not compare this single patient with the control group (laminin serum concentration in this stage: 143 ng/ml). As the degree of liver fibrosis stage increased, there was a gradual rise in basal serum laminin concentrations at entry ($rS=0.788$, p -value <0.001 , Figure 2).

After the beginning of treatment, a decrease in serum laminin concentrations was observed. We compared serum laminin concentrations of patients after six months of treatment with the basal laminin concentrations (at entry) of each fibrosis stage. Although there was a gradual decrease in serum laminin concentrations of progressive fibrosis stages (i.e. Stages 4 and 5), differences were not statistically significant ($p < 0.093$ and < 0.054 , respectively). Conversely in early stages of fibrosis (i.e. Stages 0-3) differences between the serum laminin concentrations at entry compared with the laminin concentrations after the beginning of treatment were statistically significant (for Stages 0 and 1, $p < 0.001$; and for Stages 2 and 3, $p < 0.05$).

Table 3 shows the cutoff point, sensitivity, and specificity of serum laminin concentrations. In this table the ROC curve data, for patients before and after the treatment, are presented. Figure 4 illustrates the ROC curve of serum laminin concentrations in patients (at entry) for differentiation of patients with liver fibrosis vs. control group.

Discussion

Several serum markers have been developed to assess fibrogenesis and investigations have been made to replace liver biopsy with non-invasive markers of liver fibrosis, which have been assessed in many studies. However, questions remain regarding their sensitivity and significance and whether changes in concentrations of these markers during the treatment protocol can be detected (27). In our study we have assessed serum laminin concentrations in patients with chronic hepatitis in an attempt to evaluate its predictive value for the risk of fibrosis progression. In addition, we have investigated changes in serum laminin concentrations during the treatment protocol.

As shown in Table 2, the mean serum laminin concentrations in patients with chronic liver disease were significantly higher than those of healthy controls. These results are consistent with other reported studies (16, 28). As the stage of liver fibrosis increases, there is a rise in serum laminin concentrations (Table 2) and patients in a higher fibrosis stage show higher serum laminin concentrations. In addition, as is clear from Figure 2, the correlation of serum laminin concentrations with various histological stages of liver fibrosis revealed a strong positive correlation ($rS= 0.788$). This indicates a positive relationship between serum laminin concentrations and the degree of liver fibrosis, and the two variables are linearly related ($p < 0.001$). As shown in Table 2, differences in serum concentrations of laminin at various stages during the treatment protocol, compared to healthy controls, were statistically significant ($p < 0.05$). It seems that during the treatment protocol there was a decrease in serum laminin concentrations. After six months of treatment, gradual decreases in serum laminin concentrations were observed. When we compared basal serum laminin

concentrations in patients (at entry) with the concentrations of laminin after 6 months of treatment, we observed that the serum laminin concentrations did not differ statistically in stages 4 and 5 of liver fibrosis. Conversely, in the early stages of liver fibrosis (0-3) the differences were statistically significant ($p < 0.05$). Therefore, it appears that treatment is more effective in the early stages of liver fibrosis, because only in patients with a liver fibrosis score of less than 4 a decrease in serum laminin concentrations occurred after treatment. An increase in the inflammation grade of liver damage led to a rise in serum laminin concentrations, and the correlation is statistically significant (data not shown).

As shown in Table 3, a cutoff point of 52 ng laminin/ml serum was obtained for discrimination of various stages of liver fibrosis with a reasonably good sensitivity, specificity, PPV and NPV. The AUC of serum laminin ROC curve was found to be 0.974 indicating that serum laminin is a useful diagnostic index of liver fibrosis. We also used this cutoff point (52 ng/ml) for creating the ROC curve after treatment. Again, the results showed a reasonably good AUC, sensitivity, specificity, PPV and NPV. We conclude that this cutoff point is also suitable for discrimination of our patients with fibrosis during treatment.

Our data are consistent with the work of other groups who reported that serum laminin concentrations increase in chronic liver disease (28-36). Schneider et al (16) and Castera et al (37) reported that laminin concentrations increase in early stages of chronic liver disease and the highest concentrations were in active cirrhosis and chronic active hepatitis.

Several mechanisms are proposed for the elevation of serum laminin concentrations in chronic hepatitis patients. Besides the increased production of laminin in the liver, an additional effect due to a lack of degradation of this protein by liver endothelial cells should also be considered. As demonstrated by Smedsrod et al (38), apart from an increase in tissue deposition or turnover, there would be a decrease in the liver's ability to degrade this protein. In a study of alcoholic liver disease patients, serum laminin P1-peptide concentrations were higher on admission (alcohol intake period) and rapidly returned toward the normal range through alcohol abstinence (39).

In our study we observed that after the beginning of treatment a gradual decrease occurred in serum laminin concentrations. Possibly treatment of liver fibrosis causes the liver endothelial cells to regenerate and new endothelial cells degrade this glycoprotein. Further studies are needed to gain a complete understanding of laminin metabolism.

In conclusion, the findings of our study suggest that serum laminin is a useful non-invasive marker of liver fibrosis. There was a strong positive correlation between serum laminin concentrations and the degree of liver fibrosis and inflammation (all stages and grades). Serum laminin concentrations may also be used for the follow-up of liver fibrosis in patients with chronic liver disease as well as for the assessment of liver fibrosis where liver biopsy is contraindicated.

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Table 3. ROC curve of laminin serum level for discrimination of patients with liver fibrosis vs. control group (laminin cutoff point =52 ng/ml) before and after treatment.

Stages of sampling	AUC (95% CI)	P	Sensitivity	Specificity	PPV	NPV
Before treatment	0.974 (0.945 -1.004)	<0.001	96.8%	80%	93.7%	88.8%
After treatment	0.926 (0.837-0.980)	<0.001	83.9%	80%	92.8%	61.5%

AUC: area under the curve. 95% CI: 95% confidence interval. PPV: positive predictive value. NPV: negative predictive value.

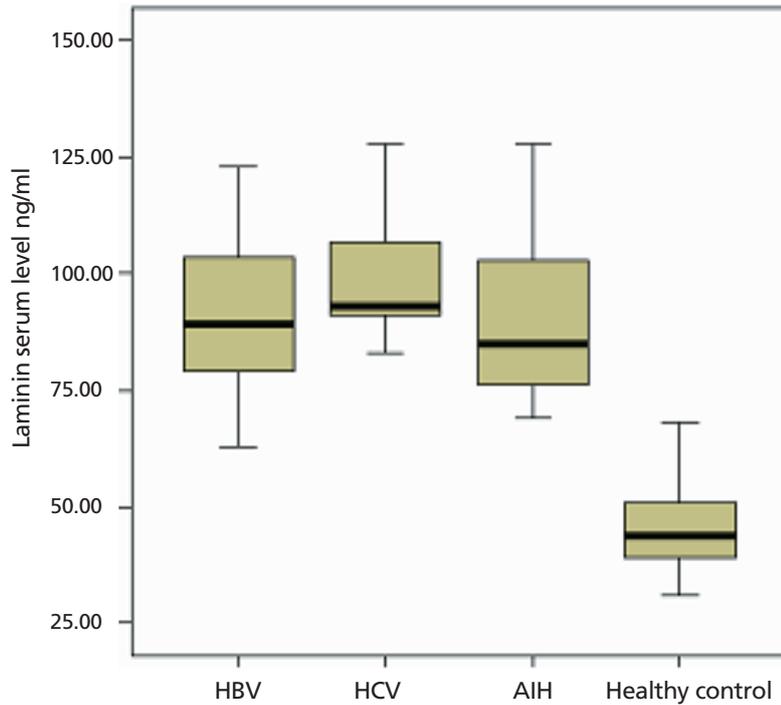


Figure 1. Box plot for serum laminin concentrations (ng/ml) in patients with hepatitis B virus (HBV), hepatitis C virus (HCV), autoimmune hepatitis (AIH) and control groups, at entry.

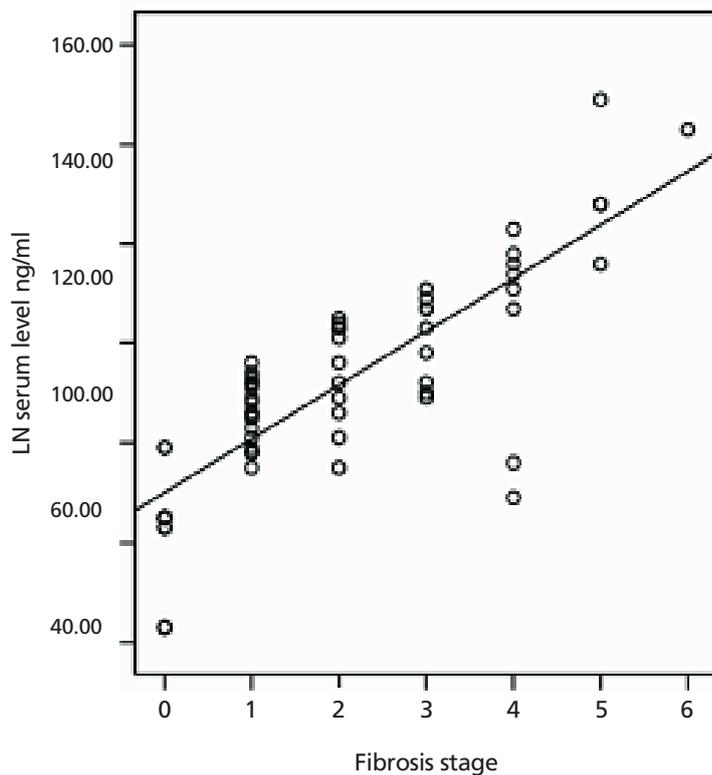


Figure 2. Correlation between serum laminin concentrations (at entry) and stages of fibrosis in liver biopsies ($r_s=0.788$, $p < 0.001$).

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