

• VIRAL HEPATITIS •

Effect of lamivudine treatment on plasma levels of transforming growth factor β_1 , tissue inhibitor of metalloproteinases-1 and metalloproteinase-1 in patients with chronic hepatitis B

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Abstract

AIM: Transforming growth factor (TGF)- β_1 , metalloproteinase (MMP)-1 and its tissue inhibitor (TIMP)-1 are considered predictive biomarkers of chronic hepatitis activity and fibrosis. The aim of this study was to evaluate the effect of lamivudine treatment on the plasma levels of these peptides in patients with chronic hepatitis B.

METHODS: TGF- β_1 , MMP-1 and TIMP-1 plasma concentrations were measured with an enzyme immunoassay in 40 patients treated with lamivudine for 48 wk. Elimination of HBV-DNA and HBV antigens was evaluated 24 wk after treatment completion.

RESULTS: Baseline TGF- β_1 (29.6 \pm 2.2 ng/mL) and TIMP-1 (1 578 \pm 93 ng/mL) significantly exceeded normal values (18.3 \pm 1.6 ng/mL and 1 102 \pm 67 ng/mL respectively). Lamivudine treatment resulted in a significant decrease of TGF- β_1 and TIMP-1 during treatment with an increase after 24 wk of treatment. Pretreatment MMP-1 levels (6.7 \pm 0.7 ng/mL) were significantly lower than normal values (11.9 \pm 0.9 ng/mL) and increased during treatment and follow-up. A significant correlation was noted between TGF- β_1 or TIMP-1 and aminotransferases as well as fibrosis scored in liver biopsy specimens. There were no statistically significant differences of TGF- β_1 , TIMP-1 and MMP-1 between four groups at baseline, 24 and 48 wk of treatment. TGF- β_1 and TIMP-1 levels increased significantly in non-responders and normalized in responders at wk 72. MMP-1 also normalized in responders and decreased to values significantly lower than normal in non-responders.

CONCLUSION: These findings support the role of TGF- β_1 , TIMP-1 and MMP-1 in the pathogenesis of chronic hepatitis B. Because of their association with hepatic injury and antiviral treatment efficacy, determination of these peptides may be useful in disease management.

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INTRODUCTION

Transforming growth factor- β_1 (TGF- β_1) is considered a pivotal inducer of liver fibrosis, acting mostly through activation of hepatic stellate cells (HSCs), which are the main source of extracellular matrix (ECM) proteins^[1-3]. The effect of TGF- β_1 on liver fibrosis is at least in part related to stimulation of the tissue inhibitor of metalloproteinases-1 (TIMP-1), which affects metalloproteinases (MMP) activity and is responsible for inhibition of ECM proteins breakdown^[4]. Apart from a profibrogenic role, TGF- β_1 inhibits DNA synthesis serving as a terminator of regenerative cell proliferation and induces apoptosis of hepatocytes in normal liver and during regression of liver hyperplasia^[5]. On the other hand, TGF- β may inhibit stellate cells apoptosis and promote their survival, which are at least in part a result of anti-apoptotic effect of TIMP-1^[6]. Additionally TGF- β_1 exerts regulatory, mostly immunosuppressive effects on the immune system. Since hepatitis B virus (HBV) infection is related to an immune response, cell proliferation and fibrosis, modulation of TGF- β_1 might affect the course of chronic viral hepatitis B^[7,8]. The possible role of TGF- β_1 , TIMP-1 and MMP-1 as predictive biomarkers of chronic hepatitis activity and progression was supported by recent clinical studies^[9-16], which demonstrated their association with hepatic function impairment or fibrosis, but did not evaluate their effect of antiviral treatment. We undertook this study to evaluate the effect of lamivudine, the most widespread antiviral medication for chronic HBV infection, on plasma TGF- β_1 , TIMP-1 and MMP-1 levels in patients with chronic hepatitis B.

MATERIALS AND METHODS

Patients

Ethical approval for the study was obtained from the Bioethical Committee of the Medical Academy of Bialystok. Informed consent was obtained from 40 patients (13 females and 27 males, mean age: 45 \pm 3 years) with chronic hepatitis B, who were included into the protocol of lamivudine (ZeffixTM, Glaxo-Smith-Kline) treatment. Normal values of TGF- β_1 , TIMP-1 and MMP-1 were collected from 13 healthy volunteers (5 females and 7 males, mean age: 47 \pm 2 years). The diagnosis of chronic hepatitis B was confirmed by the presence of HBs and HBe antigens with stable elevated alanine aminotransferase (ALT) activity for at least 6 mo. Additionally the disease activity was confirmed by the presence of viral replication and evaluation of liver biopsy specimens performed by means of the Hepafix System (Braun, Melsungen, Germany). Paraffin-embedded biopsy specimens were stained and evaluated using the Scheuer scoring system^[17]. Patients received 100 mg of lamivudine daily for 48 wk. Plasma levels of TGF- β_1 , TIMP-1 and MMP-1 were measured before treatment and at wk 24, 48 (end of the treatment) and 72. These results were compared to standard laboratory indices of liver injury. To evaluate treatment efficacy patients were divided into four groups with respect to elimination of HBV antigens and HBV-DNA at week 72 (24 wk after

completion of treatment). Criteria for inclusion into particular groups were as follows: group I (complete response): HBsAg (-), HBeAg (-), HBV-DNA (-); group II: HBsAg (+), HBeAg (-), HBV-DNA (-); group III: HBsAg (+), HBeAg (-), HBV-DNA (+); group IV (no response): HBsAg (+), HBeAg (+), HBV-DNA (+).

Methods

Venous blood for plasma TGF- β_1 , TIMP-1 and MMP-1 was collected on ice using tubes with EDTA. Samples for TGF- β_1 were immediately activated with acetic acid and urea and assayed with ELISA using recombinant human TGF- β soluble receptor type II (T β RII) as a solid phase precoated onto a microplate (Quantikine[®], R&D Systems Inc., Minneapolis, USA) as we described previously^[18]. TIMP-1 and MMP-1 were assayed by the two-site ELISA sandwich technique (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England) using specific antibodies as a solid phase. MMP-1 assay recognises total human MMP-1, ie. free and complexed with TIMP-1. TIMP-1 assay recognises total human TIMP-1, including free and complexed with any metalloproteinases bound to the solid phase. TIMP-1 or MMP-1 bound to the solid phase, were detected by peroxidase labelled antibodies. There is no cross-reactivity between TIMP-1 and MMP-1 in these assays.

Liver function tests: ALT, aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyltranspeptidase (GGT) activity and bilirubin concentration were measured using a Cobas Mira instrument (Roche) and the prothrombin index (PI) was determined using Kselmed K-3002 (Poland).

Statistics

Values were expressed as the mean \pm SE. The significance of the difference was calculated by two-tailed Student's *t* test. For correlation analysis the Pearson product moment correlation was performed. Values of $P < 0.05$ were considered as statistically significant.

RESULTS

Plasma concentrations of TGF- β_1 and TIMP-1 were determined before lamivudine treatment (mean: 29.6 \pm 2.2 ng/mL and 1 578 \pm 93 ng/mL respectively) and significantly exceeded mean normal values (18.3 \pm 1.6 ng/mL and 1 102 \pm 67 ng/mL respectively). Treatment resulted in a significant decrease by wk 24 and a further decline at the end of the treatment. However 24 wk after treatment completion mean values of both TGF- β_1 and TIMP-1 increased (Table 1). In contrast, mean MMP-1 baseline level (6.7 \pm 0.7 ng/mL) was almost half the normal value (11.9 \pm 0.9 ng/mL) and increased during treatment. After completion of treatment the levels still remained lower than normal (Table 1). There was a significant positive correlation between TGF- β_1 , TIMP-1 and ALT or AST (Table 2). A significant correlation was also demonstrated between TIMP-1 and ALP or GGT, but there was no association between MMP-1 and the biochemical indices of liver injury (Table 2). As demonstrated in Figure 1 a significant positive correlation ($r = 0.435$; $P < 0.01$) was observed between TGF- β_1 and TIMP-1. There was no correlation between TGF- β_1 and MMP-1 ($r = 0.069$), or between TIMP-1 and MMP-1 ($r = -0.143$).

As demonstrated in Table 3 scored degree of hepatic inflammation and fibrosis was similar before and after the treatment. Histologic observations in biopsies performed before the treatment showed a significant correlation between the degree of fibrosis and plasma TGF- β_1 or TIMP-1 levels (Table 4). There was no association between scored inflammation or fibrosis and classical biochemical parameters of liver injury.

Table 1 Plasma concentrations of TGF- β_1 , TIMP-1 and MMP-1 during treatment with lamivudine (mean \pm SE)

	Normal values	Time since treatment beginning (wk)			
		0	24	48	72
TGF- β_1 (ng/mL)	18.3 \pm 1.6	29.6 \pm 2.2 ¹	22.6 \pm 1.2 ²	21.9 \pm 1.5 ²	24.1 \pm 2.4
TIMP-1 (ng/mL)	1 102.0 \pm 67	1 578.0 \pm 93 ¹	1 235.0 \pm 67 ²	927.0 \pm 50 ²	1 215.0 \pm 98 ²
MMP-1 (ng/mL)	11.9 \pm 0.9	6.7 \pm 0.7 ¹	8.3 \pm 0.5 ¹	9.6 \pm 0.5 ¹	9.7 \pm 0.7 ²

Statistical significance in comparison with normal ¹ and baseline ² values.

Table 2 Correlation expressed by *r*-value and its significance between analyzed biochemical indices and TGF- β_1 , TIMP-1 or MMP-1 in chronic hepatitis B patients treated with lamivudine

	mean \pm SE	TGF- β_1	TIMP-1	MMP-1
Bil (mg%)	1.02 \pm 0.06	0.049	0.119	-0.059
ALT (U/L)	74.50 \pm 5.9	0.201 ^a	0.247 ^a	0.005
AST (U/L)	60.10 \pm 5.2	0.244 ^a	0.269 ^a	-0.029
ALP (U/L)	96.10 \pm 3.8	0.114	0.295 ^a	-0.071
GGT (U/L)	47.70 \pm 4.9	0.062	0.247 ^a	-0.102
PI (%)	87.30 \pm 1.5	-0.109	0.006	-0.065

Bil: bilirubin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; GGT: gamma glutamyltranspeptidase; PI: prothrombin index. ^a $P < 0.05$ vs other groups.

Table 3 Values of scored degree of hepatic inflammation and fibrosis in liver biopsy specimens obtained from patients before and after lamivudine treatment (mean \pm SE)

	Before the treatment	After the treatment
Portal inflammation	2.6 \pm 0.1	2.5 \pm 0.2
Lobular inflammation	1.9 \pm 0.1	1.8 \pm 0.1
Fibrosis	2.0 \pm 0.2	1.8 \pm 0.2

Table 4 Correlation expressed by *r*-value and significance between scored degree of hepatic inflammation or fibrosis and plasma levels of TGF- β_1 , TIMP-1 and MMP-1 in patients with chronic hepatitis B before lamivudine treatment

	Inflammation		Fibrosis
	Portal	Lobular	
TGF- β_1	0.207	0.179	0.391 ^a
TIMP-1	0.224	0.319	0.620 ^a
MMP-1	0.009	-0.094	-0.135

^a $P < 0.05$ vs others.

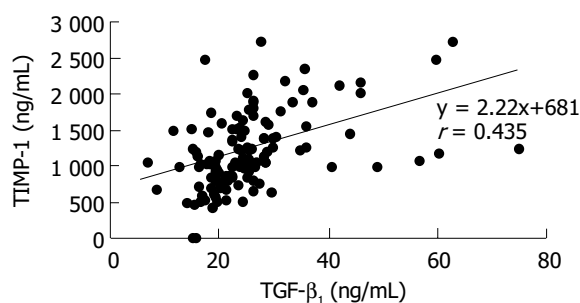
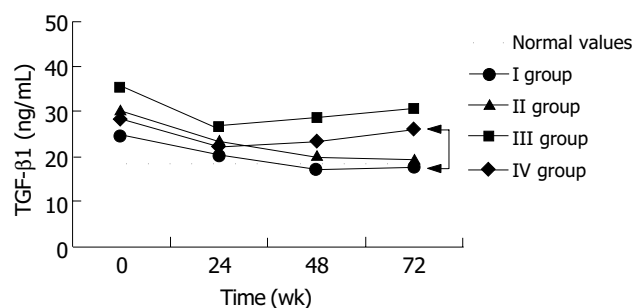
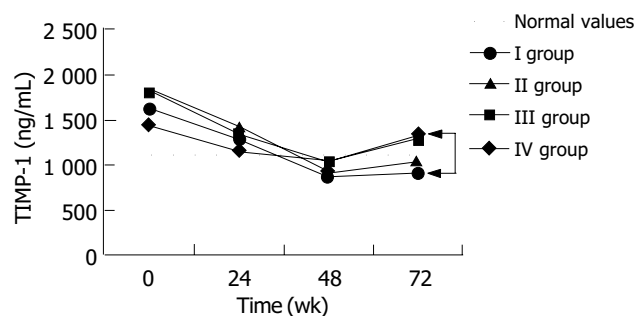
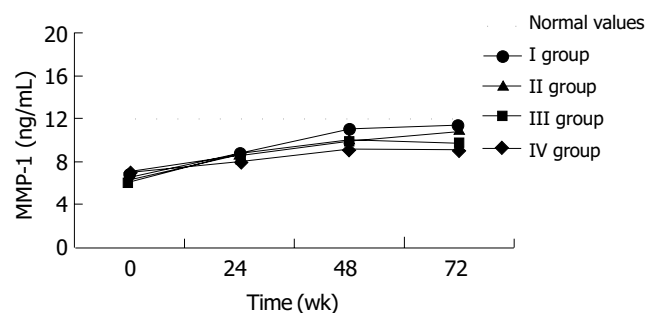
The majority of patients (23-57.5%) did not respond to treatment and were classified into group IV. A complete response (group I), with elimination of HBs and HBe antigens as well as HBV-DNA, was observed in 5 patients (12.5%). Six patients (15%) demonstrated a partial response (group II), and another six eliminated HBeAg with detectable viral replication (group III). Evaluation of baseline liver function tests and efficacy of treatment showed no statistically significant differences between the four groups. As demonstrated in Table 5, at the end of the treatment, patients in groups III and IV had significantly higher ALT activities than patients in group I. This difference was statistically significant in respect to both ALT and AST activities, 24 weeks after the treatment completion (Table 5). Histologic pictures did not demonstrate significant differences between groups in liver biopsies performed before

Table 5 Activities of alanine and aspartate aminotransferases during the treatment (0, 24 and 48 wk) and 24 wk after its completion (week 72) in four groups (mean \pm SE)

	ALT (U/L)				AST (U/L)			
	0	24	48	72	0	24	48	72
Group I	87.0 \pm 24.5	25.2 \pm 5.0	23.0 \pm 4.8	23.2 \pm 4.9	79.8 \pm 15.9	30.2 \pm 6.7	28.0 \pm 6.4	27.0 \pm 6.1
Group II	108.5 \pm 53	32.5 \pm 9.5	31.9 \pm 7.5	31.7 \pm 5.7	66.7 \pm 24.8	44.2 \pm 12.7	32.1 \pm 4.8	29.8 \pm 3.8
Group III	127.0 \pm 43.4	38.7 \pm 8.2	78.0 \pm 30.1 ^a	88.2 \pm 34 ^b	117 \pm 47.2	39.2 \pm 8.8	60.5 \pm 15.9	63.5 \pm 22 ^a
Group IV	101.0 \pm 11.8	78.6 \pm 8.3	69.2 \pm 6.3 ^b	68.5 \pm 4.2 ^b	65.9 \pm 6.4	65.2 \pm 21.2	59.7 \pm 8.4	58.7 \pm 3.9 ^a

Statistical significance in comparison with group I (complete response to treatment): ^a $P < 0.05$, ^b $P < 0.001$ vs other groups.

and after the treatment. During treatment TGF- β_1 , TIMP-1 and MMP-1 plasma levels were similar among all patients before divided into the response-related groups (Figures 2, 3, 4). And there were no statistically significant differences in their concentrations between groups at baseline, 24 and 48 weeks of treatment, either. Although at week 48 the mean TGF- β_1 in the non-responder groups (III and IV) tended to increase, whereas in groups I and II normalized, the difference was not significant (Figure 2). However 24 weeks after treatment completion (wk 72), the difference between TGF- β_1 levels in groups I and IV (17.5 \pm 0.6 and 26 \pm 1.8 ng/mL respectively) became significant ($P = 0.04$). Moreover the concentration in group IV was significantly ($P = 0.009$) higher than normal values. TIMP-1 mean concentrations were similar among all groups during the treatment period and normalized at week 48 (Figure 3). However 24 wk later, values in group IV (1 350 \pm 70 ng/mL) were significantly higher than normal, as well as that in group I (900 \pm 131 ng/mL). MMP-1 levels increased in all groups during the treatment. At wk 72, MMP-1 almost normalized in responders and slightly decreased in non-responders (Figure 4). The difference between MMP-1 concentrations in group IV after treatment completion (9.1 \pm 0.8 ng/mL) and normal values was statistically significant ($P = 0.037$).

**Figure 1** Correlation between plasma TGF- β_1 and TIMP-1 in patients with chronic hepatitis B treated with lamivudine.**Figure 2** Mean TGF- β_1 plasma concentrations before and during lamivudine therapy as well as 24 wk after its completion (wk 72) in respect to the final effect of the treatment. Statistical significance in comparison to normal values are indicated with asterisks and between groups with arrows.**Figure 3** Mean TIMP-1 plasma concentrations before and during lamivudine therapy as well as 24 wk after its completion (wk 72) in respect to the final effect of the treatment. Statistical significance in comparison to normal values are indicated with asterisks and between groups with arrows.**Figure 4** Mean MMP-1 plasma concentrations before and during lamivudine therapy as well as 24 wk after its completion (week 72) in respect to the final effect of the treatment. Statistical significance in comparison to normal values are indicated with asterisks and between groups with arrows.

DISCUSSION

The pivotal role of TGF- β_1 in fibrogenesis was finally proved in transgenic mice with an overexpression of TGF- β_1 , which caused an increase of TGF- β_1 plasma levels up to 700 ng/mL and a marked upregulation of TIMP-1 gene expression^[19-21]. Chronic liver injury leading to fibrosis displayed diminished ECM degradation mainly through TIMP induction and consequent MMP inhibition^[4]. As demonstrated recently by Nie *et al.* in the process of hepatic fibrosis, fibroblasts and myofibroblasts are the major cells that express TIMP-1 and TIMP-2, and their gene expressions correlate with degree of hepatic fibrosis.

The most important factor that affects the measurement of TGF- β_1 in human is the preparation of samples with minimal contamination from platelets which are an important source of this cytokine^[22]. The Quantikine ELISA System is recommended because of the relatively quick and simple activation with acid and urea, which disrupt the majority of TGF- β_1 complexes.

As we have demonstrated previously, TGF- β_1 correlates significantly with the degree of liver insufficiency in humans with liver cirrhosis^[18]. Moreover both TGF- β_1 and TIMP-1 measured in plasma of patients with chronic hepatitis B demonstrate a significant correlation with the degree of hepatocyte injury and fibrosis in liver biopsy specimens^[10]. We confirmed these observations in the present study. As demonstrated by Yoo *et al.*^[7] HBV antigens induce expression of TGF- β_1 in the early stages of infection. According to Lee *et al.*^[8] HBV encoded pX protein enhances transcriptional activity and response to TGF- β by amplification of Smad-mediated signaling, which can also contribute to HBV-associated liver fibrosis. Therefore the stimulatory effect of HBV antigens on TGF- β_1 seems to be an important mechanism of liver fibrosis in addition to TGF- β release caused by hepatocyte injury.

Association between circulating or tissue TGF- β_1 and liver fibrosis has also been confirmed in HCV infection^[10,11]. Recent studies of Neuman *et al.*^[23], Chen *et al.* and Lu *et al.*^[16] demonstrated a similar relationship between different chronic liver diseases including primary biliary cirrhosis and alcoholic liver disease and TGF- β_1 . Our previous study showed that the positive predictive value of TGF- β_1 plasma levels exceeding the upper normal range reached 96% for liver cirrhosis^[18]. As demonstrated recently by Zhang *et al.* serum level of TIMP-1 and TIMP-1/MMP-1 could be used as the indices for the diagnosis of hepatic fibrosis in chronic hepatitis B. According to Boeker *et al.*^[9], measurement of plasma TIMP-1 detected cirrhosis with a 100% sensitivity but lower specificity. These observations were also confirmed by the association between hepatic fibroproliferation and expression of hepatic TIMP and MMP mRNA^[12]. Walsh *et al.*^[15], who studied liver histology in patients with chronic hepatitis C, underlined the high sensitivity of TIMP-1 and TIMP-2 in detecting advanced liver disease. According to Nie *et al.*, there was a significant correlation between circulating and liver levels of TIMP-1 in cirrhotics, so its measurement in plasma might be useful in fibrosis management. These observations indicate the usefulness of both TGF- β_1 and TIMP-1 as possible early non-invasive biomarkers for liver fibrosis.

The rate of HBeAg seroconversion in our study (27.5%) was in range (18-32%) demonstrated by numerous authors in previous research with lamivudine treatment^[24-26]. In this study we confirmed the association between the degree of hepatocyte injury or liver fibrosis and plasma TGF- β_1 or TIMP-1 levels in patients with chronic hepatitis B. Aminotransferases, which are classical surrogate markers of hepatic injury, demonstrated similar association with the outcomes of the treatment. However, in contrast to TGF- β_1 and TIMP-1 their activities were not related to liver fibrosis. As the levels of TGF- β_1 , TIMP-1 and MMP-1 showed a similar change in all groups during therapy, it was unclear whether this was a direct effect of lamivudine on their expressions or an effect caused by HBV inhibition. However, their levels at 24 wk after therapy completion demonstrated their association with infection activity. This relationship was noted in respect to TGF- β_1 , which increased in group IV (non-responders) at the end of treatment. The effect on TIMP-1 was probably secondary to TGF- β_1 . The inhibitory activity of excessive TIMP-1 accumulation might be the reason for the decreased MMP-1 plasma concentration. These results are in accordance with our previous findings, demonstrating the strong association between TGF- β_1 or TIMP-1 plasma levels and scored hepatic fibrosis evaluated in biopsy specimens of patients with chronic hepatitis B and C^[10]. The findings of increased TGF- β_1 and TIMP-1 accompanied by an elevation in plasma carboxyterminal cross-linked telopeptide of type I procollagen (ICTP), indicative of type I collagen degradation. We suggested that collagenolytic mechanisms preceded TGF- β_1 /TIMP-1

dependent stimulation of liver fibrosis^[10]. Low MMP-1 plasma levels before treatment in the present study is consistent with this observation. It is also in accordance with the report by Murawaki *et al.*^[13], who demonstrated a decrease in MMP-1 concentration during histological progression of chronic hepatitis. Moreover, a significantly decreased baseline plasma MMP-1, followed by an increase during treatment, supports the role of TGF- β_1 /TIMP-1 dependent mechanism of liver fibrosis in patients with active chronic hepatitis B. Similar effects on MMP-1 and TIMP-1 were observed by Ninomiya *et al.*^[14] in patients with chronic hepatitis C who showed improvement of liver histology after treatment with interferon-alfa. Reduced progression of liver fibrosis during treatment of chronic hepatitis B with lamivudine has been demonstrated in numerous trials^[24-28]. Downregulation of TGF- β_1 /TIMP-1 causing an increase of MMP-1 activity should be considered as the probable mechanism underlying this effect of lamivudine. However, to confirm this hypothesis further research should be focused on the MMP-1 activity measurement.

These findings support the role of TIMP-1 and MMP-1 balance in the TGF- β_1 dependent mechanism of liver fibrosis related to HBV infection. The association of TIMP-1, MMP-1 and TGF- β_1 with hepatic injury and antiviral treatment efficacy suggest their possible usefulness in chronic hepatitis B management. Elevated TGF- β_1 plasma concentrations during antiviral therapy may indicate medication failure.

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