Transforming Growth Factor Beta One and Non Alcoholic Fatty Liver Disease

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Background and study aim: Hepatic steatosis reflects an imbalance between the uptake and synthesis of fatty acids by the liver and their oxidation and export. The mechanism of cell injury remains unclear. Transforming growth factor – beta 1 (TGF-β1) as a proinflammatory cytokine has become an important issue in the context of pathogenesis and progression of non alcoholic fatty liver disease (NAFLD). This study was planned to assess the value of TGF-β1 in different forms of NAFLD

Patients and Methods: This study included 62 patients; 20 patients with benign steatosis (group 1), 20 patients with non alcoholic steatohepatitis (NASH) (group 2) and 22 patients with cirrhosis (group 3), as well as 7 healthy subjects who served as a control group. Each group was subclassified according to the presence of obesity, type 2 diabetes mellitus and hypertriglyceridemia. All participants were subjected to abdominal ultrasound, ultrasound guided needle liver biopsy and routine laboratory investigations e.g. complete blood picture, liver function tests, fasting and 2 hours postprandial blood glucose and serum triglycerides.

Results: Serum TGF-β1 in the benign steatosis group was insignificantly different from the control group, while NASH and cirrhosis groups had significantly higher levels compared to control and benign steatosis groups (P<0.001). TGF-β1 in NASH group was significantly higher than in cirrhosis group (428.78 ± 117.15 vs 260.42 ± 110.22 ng/ml, P=0.032). In benign steatosis group, TGF-β1 was insignificantly different among subgroups. In NASH and cirrhotic patients, TGF-β1 was significantly higher in dyslipidemic subgroups.

Conclusion: Serum level of TGF-β1 was higher in patients with severe forms of NAFLD (NASH and cirrhosis) than in patients with benign steatosis.

INTRODUCTION

Non alcoholic fatty liver disease (NAFLD) consists of a spectrum of diseases including simple steatosis, non alcoholic steatohepatitis (NASH) and cirrhosis. NASH consists of steatosis plus inflammation, necrosis and fibrosis [1]. NAFLD affects 14-30% of the general population in United States [2]. NAFLD has been associated with multiple metabolic risk factors including, central obesity, dyslipidemia, hyper-tension, insulin resistance and type 2 diabetes mellitus [3-6]. The mechanism of cell injury in NAFLD entails that excess fatty acids in the liver induces formation of free radicals, which cause lipid peroxidation and induce proinflammatory cytokines [1]. Of the cytokines secreted as a response to cell injury, transforming growth factor – beta 1 (TGF-β1) plays the dominant role in mediating fibrosis, through its contribution to the activation of stellate cells and their production of extracellular matrix proteins [7].

Serum level of TGF-β and tissue level of TGF-β mRNA can be measured and used as diagnostic and prognostic markers for human diseases [8]. In the liver, TGF-β1 is the most abundant isoform of this family. It has many actions in the liver including: fibrogenesis, growth inhibition (of normal hepatocytes and stellate cells), mitogenesis, pro-apoptosis and chemo-attraction [9]. TGF-β1 stimulates extracellular matrix production not only by hepatic stellate cells but also by sinusoidal endothelial cells, however, its effect varies from one condition to another. In the context of hepatic regeneration, TGF-β1 is antiproliferative rather than pro-fibrogenic [10].

Different reports have linked TGF-β1 to different pathological hepatic states like cirrhosis and tumors [11-12]. Yet, to our knowledge, only scanty reports have assessed the link between TGF-β1 and different pathological spectrum of NAFLD. This study was designed to assess the value of TGF-β1 in different forms of NAFLD.

PATIENTSAND METHODS

This study had been carried out in the Departments of Internal Medicine and Clinical Pathology, Faculty of Medicine, Zagazig University, Egypt. Patients suspected to have NAFLD were included with the following criteria:

1- Those who are diabetic, obese or hyper-lipidemic with or without unexplained hepatomegaly.
2- Ultrasound-evidence of fatty liver.

Patients were excluded from the study if they had a disease process that might induce steatosis, inflammation or fibrosis **[13]**, as viral hepatitis, autoimmune hepatitis, Wilson's disease, hereditary hemochromatosis and those who have drug induced liver disease (e.g. methotrexate, tamoxifen, corticosteroids, amiodarone and synthetic estrogen). Pregnant females and patients with known history of alcohol consumption were also excluded. Out of the 295 patients who were primarily studied for being obese, diabetic or hyperlipidemic, 62 patients were included in this study, being free from exclusion criteria and fit to our inclusion criteria. The included patients were 35 females and 27 males with mean age 40.86±10.09 years (range 19-70 years). In addition, seven apparently healthy individuals, selected from the relatives of our patients, served as a control group, they were three males and four females, with mean age 49.29±6.16 years (range 44-60 years). The study was approved by our hospital ethical committee and after being informed about the purpose and procedures of the study, all participants signed an informed consent form. All patients and controls were subjected to thorough clinical evaluation. Obesity was defined as body mass index (BMI) ≥ 30.

Laboratory evaluation to all patients and controls included:

- Complete blood counts (CBC), fasting and 2 hours postprandial blood glucose, serum triglyceride level by colorimetric method, serum cholesterol by direct spectrophotometry, as well as routine liver function tests and enzymes including alanine aminotransferase (ALT) and aspartate aminotransferase (AST).
- Blood tests to fulfill exclusion criteria e.g. viral hepatitis serological markers (HCV Ab and HBsAg) by enzyme linked immunosorbent assay (ELISA) and auto-antibodies for autoimmune hepatitis including smooth muscle antibody (SMA) and liver kidney microsomal antibody (LKM).
- Serum level of TGF-β1 by ELISA.

Principle of the assay of serum TGF-β1 by ELISA [13]:

- A solid phase ELISA was used which is based on the sandwich principle. Standards and samples from patients and controls were diluted in assay buffer, acidified with HCL and then neutralized with NaOH. Afterwards, the neutralized standards and samples were added to the antibody coated microtiter wells.
- Then a monoclonal mouse anti TGF-β1 antibody, a biotinilated anti mouse IgG antibody and the streptavidin-HRP enzyme complex were incubated in succession.

Abdominal ultrasound and liver biopsy:

On ultrasonography, fatty infiltration of the liver produces a diffuse increase in echogenicity as compared with that of the kidneys. The cirrhosis was diagnosed on clinical, laboratory, ultrasound and histopathologic bases. Ultrasound guided percutaneous true-cut needle liver biopsy was performed to all patients.

Histopathological examination of the specimens:

Biopsy specimens were fixed in buffered formalin and embedded in paraffin wax. Sections were stained with Hematoxylin and Eosin for morphological evaluation, Perls' Prussian blue stain for assessment of iron loading and Masson's trichrome, for assessment of fibrosis. The specimens were analyzed by one pathologist with experience in liver pathology, with grading of steatosis, lobular inflammation, hepatocyte ballooning, NAFLD activity scoring, and fibrosis staging according to internationally agreed parameters [14]. Based on histopathologic diagnosis and classification, the included patients were assigned into three groups (Table1); 20 patients with benign steatosis (group 1), 20 patients with non alcoholic steatohepatitis (NASH) (group 2) and 22 patients with cirrhosis (group 3). Each group was further classified into three subgroups a, b and c according to the

presence of obesity, type 2 DM and hypertriglyceridemia respectively.

Statistical evaluation

Statistical analysis was done by using SPSS (Statistical Package for Social Science) version 19. The data were presented in the form of means and standard deviation, or in the form of numbers and percentages. Data were tested using the proper tests, including student's t-test, one way ANOVA and Chi-Square. Level of significance is P<0.05.

RESULTS

There were no significant differences among the studied groups as regard their sex and age distribution (Table 1).

Table (1): Demographic data of all patients and control groups.

Groups \ Demographic data		Sex				Age / year		
		Female		Male		Mean	SD	Range
		N	%	N	%			
Control group n=7		4	57.1	3	42.9	49.29	6.16	(44-60)
Group 1 (Steatosis) n=20	Obese	5	65.1	2	34.9	43.43	11.49	(30-64)
	Diabetic	4	57.1	3	42.9	50.14	9.67	(39-70)
	Hypertriglyceridemic	3	50	3	50	52.67	7.97	(37-60)
Group 2 (Steatohepatitis) n=20	Obese	6	85.7	1	14.3	44.43	14.15	(27-70)
	Diabetic	3	42.9	4	57.1	47.86	11.57	(38-70)
	Hypertriglyceridemic	3	50	3	50	41.67	13.26	(19-57)
Group 3 (Cirrhosis) n=22	Obese	6	75	2	25	45.38	10.39	(34-58)
	Diabetic	2	33.3	4	66.7	48.67	3.44	(44-52)
	Hypertriglyceridemic	3	37.5	5	62.5	47.00	4.60	(42-55)
P value		NS				NS		

Level of significance of P value: < 0.05 NS= non significant

Regarding Liver function tests and enzymes in all groups (shown in Table 2): the significantly highest bilirubin level was found in those with cirrhosis (P<0.001), the lowest albumin levels were reported among those with cirrhosis (P<0.01), the significantly highest levels of AST and ALT were seen among all patients with NASH (P<0.001) and prothrombin concentration was significantly worse among patients with cirrhosis (P< 0.001).

Table (2): Liver functions and enzymes in all patients and control groups.

Group \ Test		N	Serum bilirubin (mg/dl)		Serum albumin (gm/dl)		Serum AST (u/l)		Serum ALT (u/l)		Prothrombin concentration(%)	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control		7	0.400**	0.129	4.386*	0.522	16.00*	2.65	25.57*	6.35	107.14**	18.22
Benign steatosis	Obese	7	0.729	0.125	4.186	0.398	24.29	4.07	25.57	4.08	100.00	3.02
	Diabetic	7	0.771	0.512	4.057	0.447	20.14	6.36	20.71	5.28	97.86	5.67
	↑TG	6	0.850	3.367	4.417	0.366	19.33	1.86	22.00	3.58	96.50	6.44
Steato-hepatitis	Obese	7	0.857	0.321	4.029	0.382	43.66*	13.78	85.80*	20.89	90.43	9.31
	Diabetic	7	0.814	0.353	3.900	0.245	37.43*	7.89	76.14*	12.92	91.43	8.08
	↑TG	6	0.717	0.117	4.350	0.399	38.33*	6.25	74.17*	27.30	94.17	7.36
Cirrhosis	Obese	8	1.750**	1.281	2.800*	0.338	22.63	3.11	27.25	3.06	56.13**	10.75
	Diabetic	6	1.517**	0.733	2.633*	0.585	24.50	3.62	28.33	2.73	48.33**	14.28
	↑TG	8	2.412**	1.309	2.750*	0.342	23.50	3.93	26.50	3.42	55.38**	9.58

One way ANOVA $P < 0.05^{*}$, $P < 0.001^{**}$

In Table (3) we compared CBC components in all studied groups, and found that red blood cells count (RBCs), hemoglobin concentration and platelets count were significantly lowest among patients with cirrhosis (P<0.001).

Table 3: CBC in all patients and control groups.

Group \ Test		*N*	RBCs count (Million/cmm)		HB concentration (gm%)		WBCs (x103/cmm)		Platelet count (x103/cmm)	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control		7	5.3286**	0.3352	15.143**	1.069	7.33	2.14	183.86**	29.35
Benign steatosis	Obese	7	3.9014	0.3973	11.757	1.359	5.93	1.39	241.00	37.62
	Diabetic	7	4.0757	0.3534	11.400	2.012	5.16	1.36	244.43	15.78
	hypertriglyceridemia	6	3.8983	0.5329	11.033	1.343	4.32	0.79	219.50	67.20
Steato-hepatitis	Obese	7	4.0686	0.8291	11.443	2.501	6.72	2.56	209.29	58.27
	Diabetic	7	3.9729	0.4567	12.043	2.403	7.26	2.18	228.71	50.19
	hypertriglyceridemia	6	4.1700	0.3450	12.017	1.123	7.28	2.19	238.83	56.80
Cirrhosis	Obese	8	3.1638**	0.3497	9.775**	0.838	5.03	1.14	128.00**	68.29
	Diabetic	6	3.1133**	0.7188	8.917**	1.996	5.84	0.97	91.50**	48.85
	hypertriglyceridemia	8	3.0800**	0.5236	10.850**	1.122	6.03	1.23	140.25**	23.82

One way ANOVA P < 0.001**

In Table (4) we compared serum TGF-β1 values among NAFLD patients and controls. There were a highly significant difference among all groups (p<0.001). While, the mean serum value of TGF-β1 of the benign steatosis group was insignificantly different from the control group, the NASH and cirrhosis groups had significantly higher levels than both the control group (P<0.001) and the benign steatosis group (P<0.001).

Table (4): Serum TGF-β_1 values (ng/ml) among NAFLD patients and control groups.

Groups	N	Mean	SD	Range
Control	7	134.8	63.21	(47-198.3)
Benign steatosis	20	156.31	17.51	(120-179.2)
NASH	20	428.78	117.15	(238-1041.6)
Cirrhosis	22	260.42	110.22	(46-982.2)
One way ANOVA	P < 0.001			

Student t-test:

Control	vs	Benign steatosis	P = 0.32
Control	vs	NASH	P < 0.001
Control	vs	Cirrhosis	P = 0.009
Benign steatosis	vs	NASH	P < 0.001
Benign steatosis	vs	Cirrhosis	P = 0.031
NASH	vs	Cirrhosis	P = 0.032

Table (5) shows Serum TGF-β1 values (ng/ml) among all subgroups of different groups. Although the levels of TGF-β1 were higher in benign steatosis than the control and in obese and dyslipidemic cases more than diabetic cases of benign steatosis group, the difference doesn't reach significant level. All three subgroups of both NASH and cirrhotic patients had significantly higher TGF-β1 compared to control group (P<0.001) and the dyslipidemic group showed the highest values.

Table (5): Serum TGF-β_1 values (ng/ml) among different subgroups.

Group		N	Mean	SD	Range	One way ANOVA	Student t-test
Control		7	134.8	63.21	(47-198.3)	P>0.05	P>0.05 between any two subgroups, or between control and any subgroup.
Benign steatosis	Obese	7	160.85	13.21	(138-174)		
	Diabetic	7	145.02	19.95	(120-179.2)		
	Hypertriglyceridemic	6	164.19	14.03	(80.0-738.2)		
Control		7	134.8	63.21	(47-198.3)	P<0.001	P<0.001 (between control and any subgroups). P>0.05 (between any two subgroups).
Steato-hepatitis	Obese	7	357	146.7	(40.6-593)		
	Diabetic	7	397.1	148.2	(173.6-944)		
	Hypertriglyceridemic	6	400.1	145.1	(110-1041.6)		
Control		7	134.8	63.21	(47-198.3)	P<0.001	P<0.01 (between control and ↑TG). P<0.05 (between control vs obese or diabetics).
Cirrhosis	Obese	8	236.84	90.12	(41.4-490.9)		
	Diabetic	6	238.25	95.17	(78.7-782.2)		
	Hypertriglyceridemic	8	290.71	101.71	(70.7-982.2)		

DISCUSSION

Non alcoholic fatty liver disease is characterized by fat accumulation in the liver, which may progress to non-alcoholic steatohepatitis and cirrhosis [4]. It is becoming increasingly recognized worldwide due to its prevalence in obesity, diabetes and insulin resistance syndrome [15]. We investigated the status of TGF-β1 in NAFLD in all its pathological forms in a cross sectional way. We also evaluated it in respect to the common etiological factors for NAFLD; obesity, type 2 DM and hypertriglyceridemia. The preponderance of female sex over male was specifically higher in the obese group. Within the obese group, females tended to have benign steatosis and NASH (Table 1). This was in agreement with Luyckx et al., who identified female sex as a risk factor for NASH [16]. In our study, NASH occurred in a nearly similar frequency in both sexes in the diabetic and hypertriglyceridemic groups (Table 1). This trend was also found in another study on NASH patients by Becon and his colleagues [17]. Although NAFLD occurs in all age groups including children, we conducted our work only on adult patients. In this category, the mean age of NAFLD occurred mostly in the forties (Table 1). This was in agreement with several studies that showed the highest prevalence of NAFLD in those between 40 and 49 years of age [17-18].

Our results showed values of serum bilirubin, serum albumin and prothrombin time within normal range among different groups, except in cirrhotic patients (Table 2). Similar results were obtained by multiple studies on NAFLD patients which demonstrated that no hepatic dysfunction occurs until cirrhosis and liver failure start [18-20]. On the other hand, and going with multiple previous reports [17,19-20], ALT level was significantly higher in NASH patients than other groups (Table 2). Hence, we used high ALT level as a laboratory criteria for identifying NASH patients. RBCs count, hemoglobin concentration and platelet count were significantly lowest among patients with cirrhosis (Table 3). This was in agreement with technical review by American gastroenterological association on NAFLD that stated that hematological parameters are usually normal unless cirrhosis develop [21].

Our results showed that plasma TGF-β1 was significantly higher in NAFLD patients when compared to the control individuals. Although the TGF-β1 of the benign steatosis group was insignificantly different from the control, the NASH and cirrhosis groups had significantly higher levels when compared with either control group or benign steatosis group. Moreover, the TGF-β1 level in NASH group was higher than in cirrhosis group (Table 4). The above findings were in agreement with Yokohama et al., whose work demonstrated that blood markers of fibrosis including TGF-β1 and type IV collagen were significantly elevated in patients with NASH [22]. Our results were also supported by that obtained by Hasegawa et al., who concluded that the plasma TGF-β1 level in NASH patient was significantly elevated as compared to healthy controls and benign steatosis patients [23]. Hence, TGF-β1 could be a useful marker for diagnosis of NASH.

In our study, the dyslipidemic subgroup had significantly highest level of TGF-β1 when compared to obese and diabetic subgroups, within each patient group (Table 5). Comparative data in that field was not sufficiently tested before; however, some retrospective studies

showed high prevalence of obesity, diabetes and dyslipidemia in cryptogenic cirrhosis with or without associated hepatocellular carcinoma [24-25]. This supports the hypothesis that NASH may be an etiological factor in some of these patients. Moreover, in agreement with our results, Bugianesi et al. had identified hypertriglyceridemia (by logistic regression analysis) as the most significant independent factor in that field [25].

Zhe et al., reported that blockade of TGF-β signaling prevents liver fibrosis and dysfunction in the rat, but they recommended further future studies to avoid the unfavorable consequences, such as the inflammation and tissue necrosis observed in TGF-β1 gene-disrupted mice [26]. It was also suggested that insulin sensitizing agents like metformin, and other agents like angiotensin II receptor antagonists, ursodeoxycholic acid, gemfibrozil, N-acetyl-cysteine and α-tocopherol may have a beneficial effect in patients with NASH by lowering the serum levels of TGF-β1[27-29].

CONCLUSION

This study revealed that TGF-β1 was significantly higher in severe forms of NAFLD (NASH and cirrhosis) versus benign steatosis suggesting its role in the progression of NAFLD.

TGF-β1 assessment can be recommended as a future non invasive method for evaluation of NAFLD severity. Also, further studies are needed to evaluate the beneficial effect of TGF-β1 signaling blockade as a new therapy for NAFLD.

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