SOHAG MEDICAL JOURNAL
Vol. 21 No.2 July 2017

Role of Tissue Inhibitors of Matrix Metalloproteases-1 (TIMP-1) in Evolution of CCl_4-Induced Liver Cirrhosis in Mice.

Eman E. Abu-Deif[1], Doha S. mohamed[2], Nesreen G. Abd El-Haliem[3], Sherine A. Mohammed[4], Ashraf M. El-Badry[5]
1,2,3,4 Department of histology, Faculty of medicine, Sohaguniversity.
5 Department of surgery, Faculty of medicine, Sohag university.

Abstract

Background

Cirrhosis is a worldwide end stage liver disease. It is characterized by diffuse fibrosis and conversion of normal liver lobules into structurally abnormal nodules.

Aim of the work

This study was conducted to investigate the role of tissue inhibitors of matrix metalloproteases-1 (TIMP-1) in during the evolution of cirrhosis, and in fibers resolution after cessation of the insult.

Materials and methods

54 adult male Balb/c mice; about 2 months old were randomly divided into control and treated groups;Control:24 animals.In the treated groups; 30 animals were subcutaneously injected with CCl4 (20% # diluted in sunflower oil) in a dose of 1 ml per Kg twice weekly.6 animals of them were sacrificed72 hours after the last dose at the 4th week, 8th week, 12th week, and 16th week.6 animals were kept for two weeks without injection after the 16 weeks of CCl4 treatment.

Results

Our results indicated that TIMP-1 is involved in the process of fibrogenesis and fibers resolution in an experimental model of cirrhosis in mice.

Conclusion

TIMP-1 is involved in the process of fibrogenesis in mice which can be applied in new strategies for the treatment of liver cirrhosis.

Keywords: liver, cirrhosis, fibrosis, CCl_4.

Abbreviations; CCl_4: carbon tetrachloride, TIMP-1: tissue inhibitor of metalloproteinase-1,ECM: extracellular matrix, MMPs: matrix metalloproteases, HSCs: hepatic stellate cells.

Introduction

Liver cirrhosis is the end stage of a wide variety of chronic liver diseases and represents a common and difficult clinical challenge of worldwide importance. Hepatic fibrosis is considered a wound-healing response characterized by an imbalance in the synthesis and degradation of the extracellular matrix(ECM), which leads to accumulation of fibers in in the extracellular space. Activation of hepatic stellate cells which represent the main source of ECM is not only the key link in the development of hepatic fibrosis but also the decrease of fibers degradation by matrix metsalloproteinases(MMPs).MMPs in turn are regulated by several mechanisms including their specific inhibitors; the tissue inhibitors of metalloproteinase (TIMP). Accumulation of extracellular matrix during fibrogenesis occurs when there is

increased level of TIMP, which inhibits these collagenases (***Wang et al., 2011***).

A better understanding of the pathogenesis of liver cirrhosis would facilitate the development of more effective treatment options. Animal models are the gold standard for basic liver fibrosis and cirrhosis. They can be used to study different molecular mechanisms and pathways involved in fibrogenesis and different strategies for the treatment of liver cirrhosis (***Jang et al., 2008).***

Evidence that fibrosis and even cirrhosis are reversible has intensified interest in understanding the regulation of matrix degradation and fibrosis resolutionby TIMP.This can be applied in new therapieswhich might reverse liver cirrhosis ***(Iredale et al., 1998, and Domitrović et al., 2009).***

Materials and methods

Animals: 54 adult male Balb/c mice; about 2 months old with average weight 35 gm, were purchased from Assuit Experimental Animal Facility, Assuit University. The experiment was done in sohag university animal house. Animals were used with free access to water and chow. They were acclimatized to this environment for 5 days prior to the experiment. All procedures used in this experiment were approved with the local Ethics Committee of Sohag University, Faculty of Medicine.

Experiment design:

Animals were randomly divided into control and treated groups:

Control : 24 animals, 12 animals of the control group were subcutaneously injected with only sunflower oil twice weekly; the other 12 animals were kept without injection. 3 animals from each control subgroup were sacrificed at the 4th week, 8th week, 12th week, and 16th week.

In the treated groups; 30 animals were subcutaneously injected with CCl4 (SigmaAldrich Company, Germanym, 20% diluted in sunflower oil) in a dose of 1 ml per Kg body weight twice weekly (***Vanheule et al., 2008***). 6 animals of them were sacrificed72 hours after the last dose at the 4th week, 8th week, 12th week, and 16th week.6 animals were kept for two weeks without injection after the 16 weeks of CCl_4 treatment.

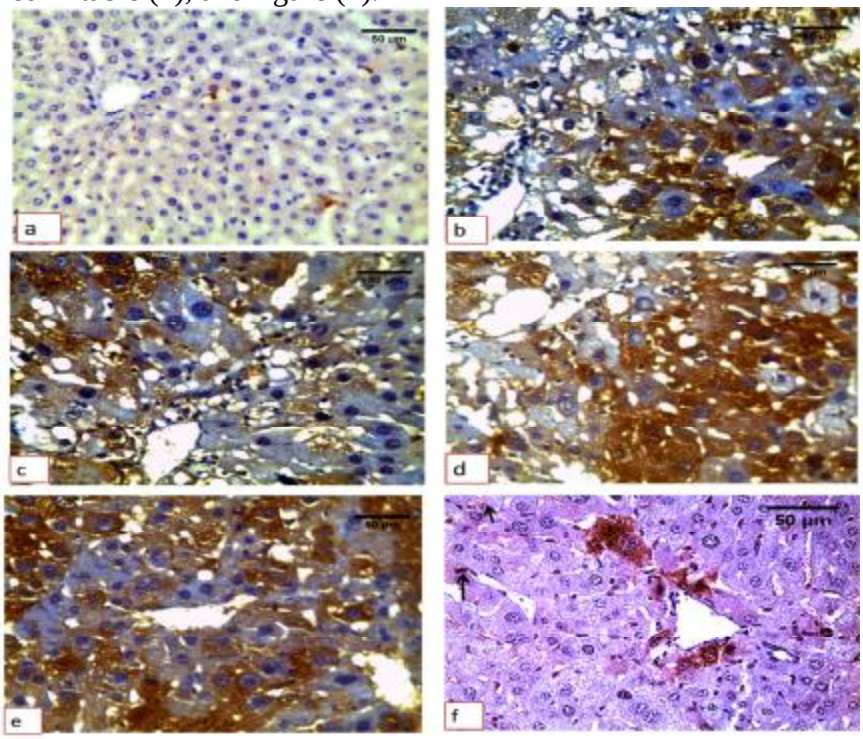
Paired sample Student *t*-test with a statistical significance of $P<0.05$ was used to analyze the data by using SPSS program version16 to detect the significance of changes between the different groups. Data were expressed as mean ± standard error(SE). All the analyses were performed in a blinded fashion**.**

Methods: Liver samples were taken for processing of paraffin sections .Formalin fixed paraffin embedded section were used for immunohistochemistry for detection of TIMP-1(Rabbit polyclonal, E3360 ; Springbioscience company, Pleasanton,1:100,), using the ultravision detection system Anti-polyvalent HRP/DAB IHC staining technique (Thermo scientific company, Neomarks, Fremont, USA.). Sections underwent deparaffinization, immersion in 0.6% hydrogen peroxide for 10 minutes , antigen retrieval by putting slides in a microwave oven at (80°C) for 10 min in 10 mM citrate buffer solution (pH 6.0). Then, sections were incubated with the primary antibody with at 4°C for 18–20h, and incubated with biotinylated secondary antibodies then with the avidin–biotin complex at room temperature, with washing in phosphate-buffered saline (pH7.2) before each incubation. The staining was visualized

with diaminobenzidine and chromogen 1: 25. The sections were finally counterstained with haematoxylin, dehydrated, cleared, and mounted (**Zaki et al.,2011).**sections underwent quantitative analysis for positive immunostaining area by means of an imaging analyzer using an imaging system constituted by a Leica digital camera connected to a light microscope Leica ICC50 Wetzlar (Germany) at the Histology Department, Faculty of Medicine, Sohag University, ten non overlapping high power fields (x400) for each case in all groups were randomly taken, and analysis of each field using Image J software (***Abdelmegeed et al.,2017***). Quantification was estimated by the percentage of stained area in comparison with the total area of fields examined.

Results

In all the control subgroups,the examined liver sections showed minimal TIMP-1 positive immunostaining in the form of brownish cytoplasmic stainig in only few kupffer cells and no immunostaining in hepatocytes. In the treated groups, TIMP-1 expressed in hepatocytes and in some Kupffer cells as shown in figure(1). There was significant increase in the mean percentage area of its expression versus the control and in between the treated groups which was progressive with the increase of the duration of CCl4 treatment then the mean significantly decreased two weeks after stop CCl4 versus the cirrhotic group(16th week) but still significantly higher than that of the control. The mean percentage areas of TIMP-1 expression in different groups of the experiment were summarized in table (1), and figure (2).



Figure(1): photomicrographs of liver section from: control animal showing TIMP-1 cytoplasmic immunopositivity in few Kupffer cells(**a)**. with CCl_4 treatment immunopositive hepatocytes with their number gradually increases from one group to the next at 4^{th} , 8^{th} ,12^{th}, 16^{th} weeks(**b,c,d,e**)

respectively,then regress to appear in few hepatocytes and Kupffer cells (arrow) after stop CCl_4**(f).**

Table(1): Mean percentage area of TIMP-1 immunoexpression.

Group	TIMP-1 immunoexpression(mean±SE)
Control	0.2210(±0.1) *
4th week	4.1333(±1.1) *
8th week	8.2667(±1.1) *
12th week	15.5333(±1.8) *
16th week	25.9333(±1.9) *
Stop CCl_4	2.3311(±1.1) *

*Statistically significant (P value<0.05).

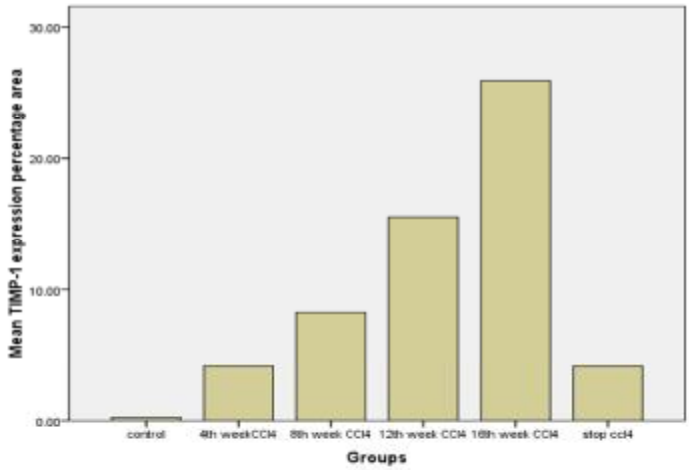


Figure (2): Mean percentage area of TIMP-1 immunoexpression

Discussion

Hepatic fibrosis is considered a wound-healing response characterized by an increase in the synthesis and a decrease in the degradation of the extracellular matrix (ECM). Degradation of extracellular a group of collagenases called matrix metalloproteinases (MMPs), produced by hepatic stellate cells (HSCs). MMPs in turn are regulated matrix protein is normally regulated by by several mechanisms including their specific inhibitors; the tissue inhibitors of metalloproteinase (TIMP), which is only secreted by non-expression increased in liver fibrosis model in rats in the form of strong positive expression in the cytoplasm of hepatocytes and myofibroblasts. This is parenchymal cells in the normal liver. Accumulation of extracellular matrix during fibrogenesis occurs when there is increased level of TIMP, which inhibits these collagenases (***Wang et al., 2011***). In our study, we found strong TIMP-1 expression in hepatocytes, and some non-parenchymal cells with significant increase in the mean percentage area of TIMP-1 expression which was progressive with the increase of the duration of CCl_4 treatment. ***Liu et al. (2005***) supported our findings; they found that TIMP-1

also consistent with the studies of ***Nie et al. (2004***) who observed increased expression of TIMP-1 and TIMP-2 in rats with hepatic fibrosis. ***Robert et al.***

(2016) found in his studies on the human HSCs in culture that the imbalance between MMP and TIMP was an important mechanism in development of fibrosis . Liver-specific overexpression of TIMP-1 led to more severe fibrosis without a significant effect on collagen synthesis (***Yoshiji et al., 2002***). However, in S. mansoni infection, deficiency of TIMP-1 or TIMP-2 did not seem to affect liver fibrosis (***Vaillant et al., 2001***). In contrast to its role in liver fibrosis, TIMP-1 did not affect the development or extent of lung fibrosis in response to bleomycin (***Kim et al., 2005***).

In our studies, we found that, 2 weeks after cessation of CCl_4 administration;there was increased degradation of collagen fibers which was revealed in our study by the significant reduction of the inhibitor of collagenases; TIMP-1 expression compared to that in that in the cirrhotic group, being demonstrated only in few hepatocytes. Our results were confirmed by previous reports which proved that the key events in the reversion of liver fibrosis include decreased active HSCs, decreased expression of TIMPs, and increased degradation of collagen (***Woessner, 1991***). Other reports confirmed that hepatic myofibroblasts derived from hepatic stellate cells undergo apoptosis during the spontaneous regression of CCl_4 induced liver fibrosis ***(Iredale et al., 1998.*** In constrast to our results, ***Domitrović et al.,(2009)*** showed that CCl_4 intoxication decreased MMP-9 expression, with further decrease 2 weeks after withdrawal of CCl_4 which allowed persistence of fibrosis in their model. They reported expression of α-SMA in hepatocytes adjacent to the fibrous septa after cessation of CCl4 treatment***. Kang et al., (2005)*** also reported persistent fibrosis in their model after withdrawal of the insult with change of the localization of α-SMA positive myofibroblast; being demonstrated only around the cirrhotic nodules.

In conclusion ;TIMP-1 is involved in the process of fibrogenesis in a CCl_4 induced model of liver cirrhosis in mice which can be applied in new strategies for the treatment of liver cirrhosis.

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