Thrombospondin-1 expression correlates with angiogenesis in experimental cirrhosis

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Abstract

AIM: To investigate the significance of Thrombospondin-1 (TSP-1) expression and its relationship with angiogenesis during experimental fibrosis.

METHODS: Cirrhosis was induced in male Wistar rats by intraperitoneal administration of diethyl nitrosamine (DEN). The serial sections from liver tissues were stained with anti-CD34 and anti-TSP-1 antibodies before being quantitated by light microscopy.

RESULTS: Our results showed that of TSP-1 expression gradually increases according to the severity of fibrosis (Group I vs group II, Group III and Group IV; Group II vs group III and group IV; group III vs group IV, P < 0.05). Moreover, TSP-1 expression was found to be correlated with angiogenesis (P < 0.05).

CONCLUSION: The correlative evidence of the link between TSP-1 and fibrosis or angiogenesis provided by this study suggests that besides its role as a strong promoter of transforming growth factor-β1 (TGF-β1), TSP-1 might have an additional role in liver fibrogenesis by stimulating angiogenesis and this protein could be a potential target to prevent fibrogenesis in chronic inflammatory diseases of the liver.

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Key words: Experimental liver cirrhosis; Immunohistochemistry; Liver fibrosis; Pathologic angiogenesis; Thrombospondin-1

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INTRODUCTION

Hepatic angiogenesis is frequently associated with inflammation and fibrogenesis during chronic liver injury[1-3]. Currently, it is not clear whether this process plays a beneficial role in the maintenance of homeostasis or contributes to liver damage during chronic inflammation. However, the fact that chronic inflammatory liver diseases respond poorly to immunosuppressive and anti-inflammatory therapy suggests that angiogenesis might be a promising therapeutic target in the prevention of fibrosis[4-8]. For this reason, attempts are being directed to evaluate the cellular and molecular mechanisms involved in the development of hepatic angiogenesis during chronic liver injury[3,4].

Thrombospondin 1 (TSP-1), one of the five members of the Thrombospondin gene family, is a matrix protein involved in complex processes including wound healing and angiogenesis[9,10]. The exact role of TSP-1 in angiogenesis is still controversial. TSP-1 can function as an inhibitor or as a promoter of angiogenesis, indicating that it might modulate this process in opposite directions[11-16].

In malignant and premalignant conditions of the liver, TSP-1 expression and its association with angiogenesis have been demonstrated[15-18]. Regarding non-neoplastic liver diseases, although the association of TSP-1 with latent transforming growth factor-β1 (TGF-β1) has been demonstrated in a few studies, the relationship between TSP-1 and angiogenesis during liver fibrogenesis has not been documented[19-21].

Therefore, this study was undertaken to investigate the significance of TSP-1 expression during diethyl nitrosamine (DEN) induced experimental liver fibrosis and to evaluate whether any relationship exists between TSP-1 expression and angiogenesis.

MATERIALS AND METHODS

Materials

This animal study was approved by the local animal ethics committee of the Akdeniz University. Male adult Wistar rats weighing 250 g were used. They were maintained on
a commercial diet and water in a room at 22 ± 2°C under normal laboratory lighting conditions.

Methods

Animal model: The rats received intra-peritoneal injections of DEN (Sigma, Saint Quentin Fallavier, France) at 100 mg/kg of body weight (n=29) or 0.9% sodium chloride (n=8) once a week. The injections were performed for 2 (n=4), 4 (n=5), 5 (n=5), 6 (n=5), 8 (n=5) and 10 (n=5) wk. The animals were sacrificed 2 wk after the last administrations and a hepatectomy was performed. Liver tissue samples were either frozen immediately in liquid nitrogen and stored at -70°C or fixed in 10% buffered formalin and embedded in paraffin.

Histology and immunohistochemistry: Four micrometer thick serial sections from the liver tissues originally fixed in formalin and embedded in paraffin were prepared and stained with hematoxylin and eosin for the histopathological assessment. Masson trichrome staining was used in the evaluation of the extent of liver fibrosis.

Immunolabeling was performed using polyclonal antibodies directed anti rat CD34 (sc-7045 goat, dilution: 1:500, Santa Cruz, CA, USA) and thrombospondin-1 (sc-12312 goat, IgG, dilution 1:200, Santa Cruz, CA, USA). An avidin-biotin-peroxidase technique (sc-2023, anti-goat ABC staining Kit; Santa Cruz, CA, USA) was used in the evaluation of the extent of liver fibrosis. An avidin-biotin-peroxidase technique (sc-2023, anti-goat ABC staining Kit; Santa Cruz, CA, USA) was used in the evaluation of the extent of liver fibrosis. Immunolabeling was performed using polyclonal antibodies directed anti rat CD34 (sc-7045 goat, dilution: 1:500, Santa Cruz, CA, USA) and thrombospondin-1 (sc-12312 goat, IgG, dilution 1:200, Santa Cruz, CA, USA). An avidin-biotin-peroxidase technique (sc-2023, anti-goat ABC staining Kit; Santa Cruz, CA, USA) was used in the evaluation of the extent of liver fibrosis.

For quantitative evaluation of TSP-1 expression, in each section positive and negative cells were counted in systematically randomly selected 10 to 15 microscopic fields by using an ocular grid at high magnification (× 400). The positive staining was calculated as the percentage of positive cells to total number of counted cells. Positive cells touching the left and lower edge of the grid were not included.

RESULTS

In this study, fibrogenesis was not observed in the control group. In DEN treated rats, fibrous septa were detected after 5 wk. The liver was cirrhotic in all cases after 8 wk. According to the severity of fibrosis, cases were divided in following groups: Group I: normal livers, group II: non-fibrotic livers (2 and 4 wk), group III: fibrotic livers (5 and 6 wk) and group IV: cirrhotic livers (8 and 10 wk) (Figure 1A). In group I, CD34 staining was restricted to the endothelium of portal vessels. While in non-fibrotic livers CD34 expression was noted in a few vascular structures around portal areas, numerous CD34-labeled vessels were detected in fibrotic livers. In group IV, CD34 staining revealed a dense vascular plexus surrounding the cirrhotic nodules (Figure 1B). Parallel to this finding, VD values were increased together with the progression of fibrosis (Figure 2). DEN-treated cases (group II, III and IV) had higher VD than the control group (P < 0.05). The difference between VD values of group II, III and IV was also statistically significant (P < 0.05) (Figure 2 and Table 1).

In normal livers (group I), TSP-1 expression was restricted to the endothelium of portal vessels and to a few hepatocytes (Figure 1C). However, in non-fibrotic group TSP-1 expression was higher than normal livers with more positive hepatocytes and perisinusoidal cells (P < 0.05). TSP-1 expression continued to increase in fibrotic livers and was more widespread in cirrhotic livers. The expression of TSP-1 in DEN-treated rat groups was significantly different from each other (P < 0.05) (Figure 1C, Figure 2 and Table 1).

| Group     | VD' mean ± SD | Median | Ranges | TSP-1 expression (%) mean ± SD | Median | Ranges |
|-----------|--------------|--------|--------|-------------------------------|--------|--------|
| I (n=8)   | 3.24 ± 1.41  | 3      | 2-6    | 1.63 ± 1.06                   | 1.5    | 0-3    |
| II (n=9)  | 5.22 ± 1.86  | 5      | 2-8    | 5.89 ± 1.18                   | 4      | 0-14   |
| III (n=10)| 9 ± 4.57     | 6      | 1-16   | 16.3 ± 7.32                   | 9      | 2-26   |
| IV (n=10) | 14.5 ± 5.97  | 11     | 8-26   | 68.5 ± 19.73                  | 44     | 0-95   |

n: Number of cases; VD: Vascular density; SD: Standard deviation; *P < 0.05.
Friedman test showed that there was a significant correlation between VD and TSP-1 expression ($P < 0.05$).

**DISCUSSION**

Results of the recent studies emphasized that hepatic angiogenesis is associated with fibrogenesis in the wound healing response to chronic liver injury\cite{1-5}. In our study, parallel to this finding, angiogenesis, assessed as VD, was increased with the progression of fibrosis ($P < 0.05$). Besides, in group II, despite the absence of overt fibrosis, VD was higher than that of normal livers, suggesting that angiogenesis is an early event which might take place before the onset of fibrosis during chronic liver damage.

It is well known that angiogenesis does not involve a single pathway, but is a complex event regulated by many angiogenic and antiangiogenic factors, including TSP-1\cite{9,10}. In neoplastic and premalignant conditions of the liver, the relationship between TSP-1 expression and angiogenesis has been studied\cite{15-17}. However, in non-neoplastic liver diseases the association of TSP-1 expression with angiogenesis and its role in this complex event has not
been documented. Because TSP-1 is also a known activator of TGF-β1, a key mediator in tissue fibrogenesis, a few studies have been focused to evaluate the effect of TSP-1 in hepatic activation of TGF-β1[19-21]. It was concluded that TSP-1 may act in the pathogenesis of liver fibrogenesis as a strong promoter of TGF-β1. Although in the present study TGF-β1 expression was not evaluated, we observed an increase of TSP-1 expression parallel to the severity of fibrosis. TSP-1 expression of normal livers was restricted to the endothelium of portal vessels and to a few hepatocytes. However, this value was 3.61 fold higher in non-fibrotic group. The percentage of TSP-1 expressing cells continued to increase in fibrotic and cirrhotic livers (P < 0.05). The present data support the contribution of TSP-1 expression in the wound healing response to chronic liver injury[19-21]. Moreover, in this study, a strong correlation between TSP-1 expression and angiogenesis was observed (P < 0.05). This finding suggests that TSP-1 is not only involved in fibrogenesis by the hepatic activation of TGF-β1 but also might play another role in the remodeling of the liver architecture by contributing to the development of angiogenesis.

Our results showed TSP-1 might be a stimulator of angiogenesis during liver injury. TSP-1 is generally recognized as an antiangiogenic agent[11,12,15]. However results of the some studies have been demonstrated that TSP-1 might be a stimulator of angiogenesis[13,14-16]. For this reason the actual role played by TSP-1 in angiogenesis has been investigated in previous studies with several different conclusions. Some studies demonstrated that the effect of TSP-1 may depend on its concentration[15,16,17], the type of domain being activated[25] and the type of receptors present on endothelial cells[26]. It has been also speculated that the actual role of TSP-1 might be related to number of its receptors[27]. Although it is not possible to conclude, based on our findings, which factor determines the angiogenic effect of TSP-1 during chronic liver injury, our data reinforce its dual role in the modulation of angiogenesis in opposite directions.

In conclusion, the results of this descriptive study reveal that in experimental liver fibrogenesis TSP-1 expression gradually increases according to the severity of fibrosis and strongly correlates with angiogenesis. Our data suggest that TSP-1 might contribute to the wound healing response to liver injury not only as a strong promoter of TGF-β1, but also as an inducer of angiogenesis and could be a potential target in the manipulation of angiogenesis in chronic inflammatory liver diseases ending with cirrhosis.

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Research frontiers
At present it is not possible to ascertain the exact pathogenic role of angiogenesis in liver fibrogenesis. However, the fact that chronic liver diseases respond poorly to conventional therapies suggests that manipulation of angiogenesis could be a promising approach to treatment. For this reason, the cellular and molecular mechanisms that are involved in the development of angiogenesis during liver fibrogenesis have been a topic of intensive investigations in the recent years.

Innovations and breakthroughs
This study demonstrated that in experimental liver fibrogenesis TSP-1 expression gradually increases according to the severity of fibrosis and strongly correlates with angiogenesis.

Applications
Based on the results of this research, TSP-1 might contribute to the wound healing response to liver injury not only as a strong promoter of TGF-β1, but also as an inducer of angiogenesis and could be a potential target in the manipulation of angiogenesis in chronic inflammatory liver diseases ending with cirrhosis.

Terminology
TSP-1 is a high molecular weight glycoprotein (450 kDa) which is composed of three identical subunits cross-linked by disulfide bonds. Each subunit is composed of several domains interacting with different surface receptors. TSP-1 is involved in various processes such as cell motility, inflammation and wound healing. It also modulates endothelial cell adhesion, motility and growth.

Peer review
This is quite an interesting investigational paper. This study demonstrated that in experimental liver fibrogenesis, TSP-1 expression gradually increases according to the severity of fibrosis and strongly correlates with angiogenesis. Further study would focus on evaluating the mechanism of TSP-1 in hepatic angiogenesis.
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