Depletion of Thymosin β4 Promotes the Proliferation, Migration, and Activation of Human Hepatic Stellate Cells

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Key Words
Thymosin β4 • Liver fibrosis • Hepatic stellate cell • Proliferation • Migration

Abstract
Background & Aims: It has recently been reported that thymosin beta-4 (Tβ4) has antifibrogenic effects in human hepatic stellate cells (HSCs) in vitro, but the mechanisms underlying these effects remain unclear. The aim of this study was to investigate the roles of Tβ4 in the proliferation, migration, and activation of HSCs. Methods: Enzyme-linked immunosorbent assays (ELISA), immunohistochemistry, and western blot assays were utilized to determine the expression levels of Tβ4 in serum, liver tissues, and LX-2 cells. Tβ4 was depleted in LX-2 cells using small interfering RNAs (siRNAs). Cell proliferation was analyzed using cell counting kit-8 (CCK-8) viability assays, and cell migration was investigated using wound-healing and transwell migration assays. Results: The expression of Tβ4 was significantly reduced during the progression of liver fibrosis. The depletion of Tβ4 significantly promoted the proliferation and migration of LX-2 cells via the activation of the PI3K/Akt signaling pathway. The pro-migratory and pro-proliferative effects of Tβ4 depletion in LX-2 cells can be counteracted by treatment with the Akt inhibitor MK-2206. In addition, Tβ4 depletion was also associated with the activation of HSCs via the enhanced expression of α-smooth muscle actin (α-SMA) and vimentin. Conclusions: Our results suggest that Tβ4 participates in liver fibrosis by inhibiting the migration, proliferation, and activation of HSCs and that Tβ4 may be an effective target in the treatment of liver fibrosis.

Y. Xiao and C. Qu contributed equally to this work.
Introduction

Liver fibrosis, a major cause of the morbidity and mortality of chronic liver disease, is characterized by excess synthesis and deposition of extracellular matrix (ECM), which disrupts the normal architecture of the liver and leads to organ dysfunction. Advanced liver fibrosis results in cirrhosis, liver failure, and portal hypertension and often requires liver transplantation [1-3]. Although there are limited therapeutic strategies for treating liver fibrosis, we now have a better understanding of the cellular and molecular mechanisms that underlie this condition. Hepatic stellate cells (HSCs) are the primary cellular source of matrix components in patients with chronic liver disease. Therefore, HSCs play an important role in the development of liver fibrosis [4]. During liver injury, HSCs are activated by cytokines and growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor-β, which are derived from activated Kupffer cells and damaged hepatocytes. In response to fibrogenic stimuli, HSCs lose their retinoid stores, proliferate, express α-smooth muscle actin (α-SMA), and produce large amounts of extracellular matrix proteins including type I collagen [5]. The human HSC cell line LX-2 was used in this study because of its similarity to primary human stellate cells; LX-2 cells express α-SMA, vimentin, platelet-derived growth factor receptor β (PDGF-Rβ), matrix metalloproteinase (MMP)-2, and tissue inhibitor of matrix metalloproteinase (TIMP)-2 [6]. PI3K (phosphoinositide 3-kinase) is composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit, which is recruited to and activated by the activated PDGF receptor following HSC activation and growth factor stimulation [7-9]. It has been reported that the activation of the PI3K/Akt pathway can facilitate HSC proliferation and α1 collagen expression [7, 10, 11]. In this regard, the activation of PI3K/Akt signaling significantly contributes to fibrotic progression.

Tβ4 is a member of the beta-thymosin family that is expressed in nearly all cell types and has multiple physiological and pathological functions such as wound healing and angiogenesis [12, 13]. Several studies have suggested that Tβ4 is involved in the process of fibrosis in different organs. Tβ4 has been shown to have pro-fibrotic effects in mice with ureteral obstruction [14]. However, Ac-SDKP, a degradation product of Tβ4, has anti-fibrotic activity [15]. When mediated by elevated expression of hepatocyte growth factor (HGF) and decreased PDGF-dependent phosphorylation, Tβ4 demonstrated anti-fibrogenic effects in hepatic stellate cells (HSCs) in vitro [10, 16]. Based on these studies, we further investigated whether Tβ4 can suppress the activation of HSCs and examined the molecular mechanisms of Tβ4 involvement in proliferation, migration, and activation. We first detected the expression of Tβ4 protein in the serum of cirrhosis patients and in liver specimens of bile duct ligation (BDL) rats with liver fibrosis progression. Secondly, we investigated the roles of Tβ4 in the activation of HSCs in terms of proliferation, migration, and ECM deposition. Finally, we studied the role of the PI3K/Akt signaling pathway in LX-2 cells with regard to Tβ4-mediated effects.

Materials and Methods

Materials and Specimen preparation

Tβ4 was purchased from Prospec (Rehovot, Israel). Fetal bovine serum (FBS) was purchased from Gibco (Los Angeles, CA, USA). Anti-Akt, anti-phospho-Akt, anti-Vimentin, anti-phospho-p85, anti-phospho-PDK1, anti-phospho-PTEN, anti-phospho-GSK-3β and anti-GAPDH antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). The anti-Tβ4 and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Anti-MMP-2 antibody was purchased from Abcam (New Territories, HK). The anti-α-SMA antibody was obtained from Sigma-Aldrich (MO, USA). MK-2206 was purchased from Selleck Chemicals (TX, USA). Lipofectamine RNAiMAX was obtained from Invitrogen (CA, USA). Serum was collected from 20 patients with cirrhosis and 15 healthy controls in Xinhua Hospital, Shanghai, China. The serum was collected from 20 patients with cirrhosis and 15 healthy controls in Xinhua Hospital, Shanghai, China. All of the patients provided written informed consent.
Rat bile duct ligation (BDL)

A total of 30 male Sprague-Dawley rats (200-250 g) underwent bile duct ligation surgery as described previously [17, 18] and were sacrificed after 4 or 6 weeks. The animal study was approved by the Animal Care and Use Committee of Xinhua Hospital, Shanghai, China.

Enzyme-linked immunosorbent assays (ELISA)

The Tβ4 ELISA kit was purchased from BlueGene Biotech Co., Ltd. Experiments using this kit were performed according to the protocol described by the manufacturer. The results were determined using a microplate reader at 450 nm (Molecular Devices, Beckman).

Masson’s trichrome staining

The liver tissues were collected and fixed in 10% buffered formalin and embedded in paraffin. The samples were sliced into 8 µm-thick sections. Masson’s trichrome staining was performed on the sections according to the protocol described by the manufacturer (GeneMed Scientists Inc). When using this stain, the collagen fibers will be stained blue, the nuclei will be stained black, and the background will be stained red.

Immunohistochemistry and immunofluorescence assays

Immunohistochemistry was performed with the chromogen diaminobenzidine (DAB) as described previously [19]. Briefly, the samples were incubated with xylol and descending concentrations of ethanol. Endogenous peroxidases were removed by incubation with 0.3% H₂O₂ for 15 min at room temperature (RT). After antigen retrieval, blocking was performed with 5% bovine serum albumin (BSA) for 30 min at RT. An anti-Tβ4 antibody was applied at its optimal concentration overnight in a wet chamber (at a dilution of 1:50). The slides were rinsed in phosphate-buffered saline (PBS) and incubated with the appropriate secondary antibody for 1 h at RT. Antibody binding was visualized using a liquid DAB Substrate Chromogen System (Dako, Glostrup, Denmark). The slides were rinsed in PBS and counterstained with hematoxylin. For immunofluorescence analysis, the cells were initially fixed with 4% paraformaldehyde. After blocking the cells with 3% BSA for 30 min at RT, the cells were incubated with the anti-Tβ4 antibody or anti-MMP-2 antibody (Abcam, Hongkong, Ltd) at RT for 2 h. The cells were washed three times with PBS and then incubated with the secondary antibody conjugated to FITC for 1 h at RT. The nuclei were counterstained with DAPI. The results were visualized using a fluorescence microscope (ECLIPSE, Nikon).

Cell culture and Transfection

The human hepatic stellate cell line LX-2 was cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum in a humidified 37°C atmosphere of 5% CO₂. The pooled Tβ4 siRNA duplexes were synthesized by Shanghai Genepharma Co., Ltd. The siRNA sequences were 5’-UCGAUAAGUGAAACUGAATT-3’, 5’-CUUCCAAAGAAACGAUUGATT-3’ and 5’-GAGGUUGGAUCAAGUUUAATT-3’. The siRNAs were transfected into LX-2 cells with Lipofectamine RNAiMAX.

Western blots

Western blots were performed according to standard procedures. Briefly, the protein concentrations of the lysates was determined using a bichoninic acid protein assay system (Pierce, Rockford, IL), and equal amounts of each sample were separated by electrophoresis on Novex 4-12% Tris-glycine gels and transferred onto nitrocellulose membranes using a dry blotting system (iBLOT system, Invitrogen, CA, USA). The membrane was pre-incubated for an hour in phosphate-buffered saline (PBS) containing 5% skim milk and 0.05% Tween 20 (PBS-T). After blocking, the membranes were incubated with the primary antibodies overnight at 4°C. The following antibody dilutions were used: Anti-Akt, 1:1,000 dilution; MMP-2, 1:1,000 dilution; α-SMA, 1:500 dilution; Vimentin, 1:1,000 dilution; p-p85, 1:1,000 dilution; p-PDK1, 1:1,000 dilution; p-PTEN, 1:1,000 dilution; p-GSK-3β, 1:1,000 dilution; and GAPDH, 1:1,000 dilution. The membranes were washed three times with PBS-T and then incubated with a species-appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The membranes were washed three times with PBS-T, and the bound antibody was detected with an enhanced chemiluminescence (ECL) detection kit (Thermo Scientific) using a Molecular Imager ChemiDoc™ XRS (Bio-RAD).
Wound-healing and Transwell migration assays
Confluent cells in six-well plates were scraped using a pipette tip to make a 22 mm-long wound. The cells were incubated at 37°C and allowed to migrate into the wound and then fixed. After fixation, the numbers of cells that moved into the scratched area were counted in a microscope. The migration ratio (%) was calculated by the width of the wound at 16 and 24 hours divided by the width of the wound at 0 h. Cell migration was evaluated in 24-well transwell chambers (Corning, MA, USA), as described by the manufacturer. Briefly, the lower chambers of the 24-well plate were filled with 500 μl of DMEM containing 10% FBS. A suspension of 1×10⁴ cells in 100μl of DMEM without FBS was placed into the upper compartments of the wells. The transwell chambers were incubated at 37°C in a humidified 5% CO₂ atmosphere for 24 h. The cells that had invaded the lower surface of the polycarbonate membranes (12 μm pore size) were fixed, stained with Coomassie blue, and quantified by counting 3 microscopic fields for each filter.

Co-immunoprecipitation
LX-2 lysates were precipitated with Tβ4 antibody using a Co-IP kit (Pierce, IL USA) as previously described [20]. Western blots were performed using an anti-actin polyclonal antibody (Santa Cruz Biotechnology).

Cell viability assay
A total of 3×10³ cells/well was seeded in 96-well plates 24 hours prior to treatment. The proliferation of the treated cells was determined by using Cell Counting Kit-8 assays (Dojindo, Kumamoto, Japan). The absorbance value of each well was determined at 450 nm using a microplate reader (Molecular Devices, Beckman).

Statistical Analysis
All data are reported as the mean ± S.D. Statistical significance was determined by Student’s t-tests using the SPSS19 software program when comparisons were made between two different groups.

Results

Tβ4 is down-regulated with liver fibrosis progression
A previous study has evidenced that hepatocytes were able to synthesize large amounts of Tβ4. Furthermore, a strong diffuse expression of Tβ4 was observed in the human liver [21]. However, the intracellular and extracellular roles played by Tβ4 under physiological conditions or in the context of different liver diseases are still unclear. In this study, we first

Fig. 1. The expression of Tβ4 decreases in liver fibrosis. (A) The serum levels of Tβ4 were significantly lower in patients with cirrhosis compared to healthy controls. (B) The expression of Tβ4 in rat liver was significantly lower 4 to 6 weeks after BDL and correlated with the progression of fibrosis.
examined the expression of Tβ4 in patients with cirrhosis and rats that underwent BDL. We compared the serum expression levels of Tβ4 in 17 patients with cirrhosis and 15 healthy controls. Serum Tβ4 levels were significantly lower in cirrhosis patients (1.5±0.5 ng/mL) than in healthy controls (2.1±0.4 ng/mL) (p=0.04) (Fig. 1A). We further used immunohistochemistry analysis to investigate the expression of Tβ4 in rat liver specimens from the BDL model. The degrees of rat liver fibrosis were determined by Masson’s trichrome staining after the rats were sacrificed at 4 and 6 weeks. The levels of Tβ4 were significantly lower 4 and 6 weeks after BDL and were negatively correlated with the progression of fibrosis progression (Fig. 1B).

Tβ4 depletion increased the proliferation of LX-2 cells

Because the down-regulation of serum Tβ4 was observed in patients with cirrhosis, we further investigated whether Tβ4 has biological anti-fibrosis properties. The human HSCs cell line LX-2 has been extensively used as a tool for studying human hepatic fibrosis since its establishment in 2005 [6]. In this study, we investigated whether Tβ4 had any effect on the proliferation of LX-2 cells. LX-2 cells were incubated with different concentrations of Tβ4 (0, 10, 100, and 1,000 ng/mL) for 24 hours. As illustrated in Fig. 2A, the growth of LX-2 cells was significantly inhibited after Tβ4 treatment. Furthermore, the proliferation of LX-2 cells was significantly enhanced when Tβ4 was knocked down with siRNA (Fig. 2B, C).
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Tβ4 depletion activated the PI3K/Akt signaling pathway in LX-2 cells

The phosphorylation of Akt (Ser 473 and Thr 308) can be inhibited by Tβ4 treatment. Correspondingly, Tβ4 depletion can significantly induce the phosphorylation of Akt at both sites (Fig. 2A-C). Because Akt is a downstream factor in the PI3K/Akt signaling pathway, further investigation is necessary to determine whether Tβ4 has an effect on the degree of phosphorylation of the components in this pathway. As illustrated in Fig. 2D, Tβ4 depletion increased phosphorylation of the regulatory subunit of PI3K, p85 (Tyr 458), and the kinase PDK1 (Ser 241). We further analyzed the expression and phosphorylation of PTEN, the phosphatase that prevents PI3K-mediated effects by hydrolyzing phosphatidylinositol (3, 4, 5)-trisphosphate to phosphatidylinositol 4, 5-bisphosphate; we observed that the phosphorylation of PTEN (Ser 380) was inhibited by Tβ4 depletion. Glycogen synthase kinase-3β (GSK-3β) is a well-established downstream component of the phosphatidylinositol 3-kinase (PI3K) signaling pathway. Fig. 2D shows that the phosphorylation of GSK-3β (Ser 9) was enhanced by Tβ4 depletion. To investigate whether Akt activation is essential to the Tβ4-mediated proliferative effect in LX-2 cells, LX-2 cells were treated with the Akt inhibitor MK-2206 (at concentrations of 8, 12, and 65 nM) for 24 hours prior to Tβ4 siRNA transfection and examined by morphological observation and proliferative assays [22]. The growth of LX-2 cells was significantly promoted by Tβ4 depletion compared to control (p=0.002). MK-2206 treatment decreased the viability of LX-2 cells and was accompanied by the appearance and outgrowth of neurite-like structures (Fig. 3).
Tβ4 depletion promoted the migration of LX-2 cells

In addition, we utilized scratch-wound healing and Transwell migration analysis to examine the effects of Tβ4 on the migration of LX-2 cells. As shown in Fig. 4A, wound-healing assays showed that treatment with Tβ4 peptide suppressed LX-2 cell migration, while Tβ4 depletion induced cell migration. (A, B) Wound-healing assays. (C) Transwell analysis. *, p<0.05; **, p<0.01, magnified 40×.

The mechanisms involved in Tβ4-mediated LX-2 cell migration

Tβ4 is believed to have an anti-migratory function because it can inhibit actin polymerization in vitro and in vivo and induce cytoskeletal disorganization [23, 24]. Here, we investigated whether Tβ4 can inhibit the migration of LX-2 cells by binding to actin and suppressing actin polymerization. We first used confocal immunofluorescence microscopy with cells stained for actin (red) and Tβ4 (green) to determine whether these two proteins are co-localized in LX-2 cells. As shown in Fig. 5A, Tβ4 was distributed around the nucleus in untreated cells. In LX-2 cells treated with Tβ4 siRNA, actin polymerization was evident around the cell membrane (Fig. 5A). To confirm the confocal microscopy findings, we immunoprecipitated actin using an antibody and determined the presence of Tβ4 in the immunoprecipitate analysis. As shown in Fig. 5B, actin was indeed co-immunoprecipitated with Tβ4 in LX-2 cells.

In addition to affecting actin polymerization, the dissociation of the actin-Tβ4 complex may influence metalloproteinase synthesis, which is necessary for extracellular matrix
In this study, Tβ4 depletion in LX-2 cells significantly increased cell migration and matrix metalloproteinase (MMP)-2 expression (Fig. 6C, D). We further examined whether Akt is required for the Tβ4 suppression-mediated MMP-2 expression and cell migration. As shown in Fig. 6, the cell migration that was induced by Tβ4 depletion was suppressed by treatment with MK-2206 (Fig. 6A, B).

**Tβ4 inhibits LX-2 cell activation as determined by the expression of α-SMA and vimentin**

Tβ4 has been reported to counteract fibrosis in cystic fibrosis and lung fibrosis [25, 26]. In this study, the anti-fibrogenic property of Tβ4 was investigated by examining its effect on HSC activation. LX-2 cells were treated with 1, 10, 100, 1000 ng/mL of Tβ4 or transfected with Tβ4-siRNA and then incubated for 24 hours. As illustrated in Fig. 7, Tβ4 significantly inhibits the expression of α-SMA and vimentin. In contrast, Tβ4 depletion induced the expression of α-SMA and vimentin.
both proteins, suggesting that Tβ4 can inhibit the activation of LX-2 cells by suppressing the expression of α-SMA and vimentin.

Discussion

Tβ4, a primary sequestering protein of cellular actin, is involved in many critical biological processes including angiogenesis, wound healing, inflammatory response, and cell migration [13, 27-29]. Tβ4 has been found both inside the cells and in extracellular fluids, such as blood serum, saliva, and wound fluid [30-32]. Nemolato et al. recently reported that hepatocytes were able to synthesize large amounts of Tβ4, but the intracellular roles of Tβ4 were still unclear [21]. In this study, we found that Tβ4 levels were decreased significantly in both human serum and liver tissues during liver the progression of fibrosis. However, the primary mechanism of Tβ4 in liver fibrosis remains to be fully established. HSCs, a key contributor to liver fibrosis, express HGF and its receptor c-Met [33]. Several studies have reported that HGF could ameliorate liver cirrhosis and resolve fibrotic process. This effect is partly due to the suppression of HSC proliferation and the induction of apoptosis in activated cells [34]. A recent study revealed that HGF can induce the expression of Tβ4, which down-regulated the expression of the PDGF-β receptor and thus prevented the proliferation of HSCs. Tβ4 can also upregulate the expression of HGF, thus establishing a paracrine loop [10, 16].

Recent studies showed that Tβ4 upregulates the expression of HGF, which can ameliorate liver cirrhosis and resolve fibrotic progression by suppressing HSC proliferation and inducing apoptosis in activated HSCs. In addition, the HGF-induced expression of Tβ4 can establish a paracrine loop [10, 16, 33, 34]. Here, we demonstrated that LX-2 cells strongly expressed Tβ4 and that Tβ4 depletion significantly promoted the growth of LX-2 cells by activating the PI3K/Akt signaling pathway. Akt (serine/threonine protein kinase) is a key

Fig. 7. Tβ4 depletion activates LX-2 cells. Tβ4 knockdown in LX-2 cells increased expression of α-SMA and vimentin. In contrast, Tβ4 treatment suppressed the expression of α-SMA and vimentin.

Fig. 8. Possible mechanisms involved in the Tβ4 depletion-mediated proliferation and migration of LX-2 cells. Tβ4 depletion in LX-2 cells promotes the phosphorylation of Akt, thereby inducing the migration and proliferation of LX-2 cells. In addition, Tβ4 depletion causes dissociation from actin, increases actin polymerization, and then enhances LX-2 cell migration.
kinase that regulates multiple cellular processes; it is therefore an ideal target for preventing the activation/transdifferentiation of HSCs. We found that the expression of phosphorylated Akt (Ser 473 and Thr 308) was significantly inhibited by Tβ4 administration. In addition, the Akt inhibitor MK-2206 suppressed Akt phosphorylation and thus counteracted the proliferative effects of Tβ4 depletion on LX-2 cells. Thus, Tβ4 may suppress the activation of HSCs by increasing HGF expression and disrupting PDGF/PI3K/Akt signaling. Other investigators have suggested that oxidative stress is implicated as an important molecular mechanism underlying fibrosis in the liver [35, 36]. The oxidative stress either contributes to the activation of HSCs and collagen production or stimulates hepatocyte regeneration after the administration of HGF[11, 37]. Tβ4 treatment can reduce the expression of several pro-fibrotic genes, including the connective tissue growth factor (CTGF), collagen type-1 (Col-I), and collagen type-3 (Col-III) in cardiac fibroblasts. [38] However, whether Tβ4 could prevent oxidative stress-induced HSC activation still requires further investigation. In this regard, Tβ4 inhibits HSC activation primarily by inducing HGF expression and disrupting PDGF/PI3K/Akt signaling.

Locomotion for adherent cells is a highly integrated process that is initiated by the forward extension of lamellipodia and proceeded by repeated cycles of protrusion, adhesion, and contraction [39, 40]. Tβ4 is a candidate regulator of cell protrusion with important roles in distinct protrusion-related processes such as actin polymerization and matrix metalloproteinase (MMP) expression. Interestingly, Tβ4 apparently has opposing roles in these processes. As a major sequestering protein of globular actin (G-actin), Tβ4 is believed to have an anti-migratory function because it inhibits actin polymerization in vitro and in vivo and induces cytoskeletal disorganization [23, 24]. In contrast, the activation of Akt by Tβ4 is considered to be pro-migratory [13, 41]. In this study, it was observed that Tβ4 depletion resulted in increased actin polymerization, thereby inducing LX-2 cell migration. In liver fibrosis, activated HSCs produce ECM, which provides cells with positional information and a mechanical scaffold for adhesion and migration by allowing binding to certain growth factors/cytokines, MMPs, and processing enzymes [42]. To evaluate the influence of Akt activation on HSCs migration that is mediated by Tβ4 depletion, the Akt inhibitor MK-2206 was used in this study. After MK-2206 treatment, the pro-migratory effect induced by Tβ4 depletion was significantly suppressed. Finally, we showed that Tβ4 administration could also suppress the expression of pro-fibrotic proteins, including α-SMA and vimentin. Altogether, our results suggested that Tβ4 was a potential anti-fibrogenic agent for the treatment of liver cirrhosis.

Disclosure Statement
The authors declare no conflicts of interest.

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