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Neovessel formation promotes liver fibrosis via providing latent transforming growth factor-β

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ABSTRACT

Aim: Hepatic fibrosis and angiogenesis occur in parallel during the progression of liver disease. Fibrosis promotes angiogenesis via inducing vascular endothelial growth factor (VEGF) from the activated hepatic stellate cells (HSCs). In turn, increased neovessel formation causes fibrosis, although the underlying molecular mechanism remains undetermined. In the current study, we aimed to address a role of endothelial cells (ECs) as a source of latent transforming growth factor (TGF)-β, the precursor of the most fibrogenic cytokine TGF-β.
Methods: After recombinant VEGF was administered to mice via the tail vein, hepatic angiogenesis and fibrogenesis were evaluated using immunohistochemical and biochemical analyses in addition to investigation of TGF-β activation using primary cultured HSCs and liver sinusoidal ECs (LSECs).
Results: In addition to increased hepatic levels of CD31 expression, VEGF-treated mice showed increased α-smooth muscle actin (α-SMA) expression, hepatic contents of hydroxyproline, and latency associated protein degradation products, which reflects cell surface activation of TGF-β via plasma kallikrein (PLK). Liberating the PLK-urokinase plasminogen activator receptor complex from the HSC surface by cleaving a tethering phosphatidylinositol linker with its specific phospholipase C inhibited the activating latent TGF-β present in LSEC conditioned medium and subsequent HSC activation.
Conclusion: Neovessel formation (angiogenesis) accelerates liver fibrosis at least in part via provision of latent TGF-β that activated on the surface of HSCs by PLK, thereby resultant active TGF-β stimulates the activation of HSCs.

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1. Introduction

Liver fibrosis, a common feature of almost all chronic liver diseases, is caused by the excessive accumulation of extracellular matrix (ECM) proteins, including collagen produced mainly by hepatic stellate cells (HSCs) through the process termed activation [1–3]. Under physiological conditions, quiescent HSCs embrace sinusoids as liver-specific pericytes. When the liver parenchyma is chronically injured by various causes, HSCs detach from the sinusoids and subsequently transform into myofibroblast-like cells. This HSC activation is characterized by a loss of lipid droplets, the enhanced production of ECM, and the expression of activation markers such as α-smooth muscle actin (α-SMA) [4]. The HSC activation process is regulated by both autocrine and paracrine growth factors [4,5], among which transforming growth factor (TGF)-β, the most fibrogenic cytokine, plays a critical role [6,7].

TGF-β is produced as a high molecular weight latent form with its propeptide region known as "latency associated protein (LAP)", and thereby must be activated before exerting its biological activity [8]. Latent TGF-β is activated by plasma kallikrein (PLK), which is bound to glycophosphatidylinositol-anchored urokinase-type plasminogen activator receptor (uPAR) on the cell surface and released by phosphatidylinositol-specific phospholipase C (PI-PLC) [9]. PLK cleaves LAP between R58 and L59 during liver fibrosis [10]. After cleavage, the N-terminal side LAP degradation products ending at R58 (R58 LAP-DPs) remain within the ECM of the liver tissues through LTBP, serving as a footprint for active TGF-β generation. We produced a specific antibody (anti-R58 antibody) that detects a neoepitope at the cutting edge of R58 LAP-DPs [10].

Angiogenesis in the adult liver occurs both in pathological settings, such as cirrhosis and tumor development, and in physiological conditions such as liver regeneration [11,12]. Blood vessels in the liver are classified into the hepatic artery, portal vein and sinusoidal blood vessel groups. Thus, liver sinusoidal endothelial

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cells (LSECs) are the largest population of endothelial cells in the liver.

Hepatic angiogenesis and fibrogenesis occur in parallel during liver diseases [11,13]. Sahin et al. showed that VEGF transgenic mice with increased serum VEGF concentrations have augmented liver fibrosis [14]. However, how the overproduction of VEGF induces liver fibrosis has not yet been determined.

The current study addressed a role of ECs as a source of latent TGF-β, the precursor of the most fibrogenic cytokine TGF-β.

2. Materials and methods

2.1. Materials

Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD31 monoclonal antibody (Clone 390) and rat anti-mouse CD146 monoclonal antibody (Clone ME-9F1) were purchased from Millipore (Billerica, MA, USA) and Bio Legend (San Diego, CA, USA), respectively. FITC-conjugated mouse anti-α-SMA monoclonal antibody (Clone 1A4) and anti-α-SMA monoclonal antibody (Clone 1A4) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Dako (Glostrup, Denmark), respectively. Neutralizing mouse anti-TGF-β1 monoclonal antibody (Clone 9016) and sheep anti-rat IgG magnetic bead-conjugated antibody (Cat. No.110-35) were purchased from R&D Systems (Minneapolis, MN, USA) and Invitrogen (Carlsbad, CA, USA), respectively. Recombinant VEGF 165 and PI-PLC were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Sigma–Aldrich (St. Louis, MO, USA), respectively. An R58 monoclonal antibody that recognizes neo-epitopes formed via the PLK-dependent proteolytic activation of latent TGF-β1 was produced and characterized as previously reported [10].

2.2. Animal experiments

One hundred microliters of saline with or without recombinant VEGF 165 was injected intravenously via the tail veins of three 10-week-old C57BL/6 male mice (Japan SLC Inc., Shizuoka, Japan) daily at doses of 10 or 20 ng/g body weight for 10 days. The mice were euthanized, and the livers were harvested for biochemical and immunohistochemical analyses. All animal experiments were performed in compliance with the protocols approved by the RIKEN Institutional Animal Use and Care Administrative Advisory Committee.

2.3. Staining of liver tissue sections

Liver tissues were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin, and tissue sections (6-μm thick) were prepared using a Leica sliding microtome (Leica Microsystems, Nussloch, Germany). The liver tissue sections were deparaffinized, rehydrated and incubated for 5 min with a drop of Proteinase K (Dako Envision) in 2 ml of 0.05 M Tris–HCl buffer (pH 7.5) at room temperature. Thereafter, endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide in methanol at room temperature for 10 min. The liver tissue sections were stained with Myer's hematoxylin solution and 1% Eosin Y solution (Muto Pure Chemicals, Tokyo, Japan). For CD31 staining, liver sections were incubated at 4 °C overnight with rat anti-CD31 monoclonal antibody (5 μg/ml) and thereafter with the biotinylated rabbit anti-rat IgG antibody (1:200) included in the Vectastain Elite ABC kit for 30 min at room temperature. A 3,3′-diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratories, Inc., Burlingame, CA, USA) was used for its chromogenic substrate, which develops as a brown precipitate, to visualize immunolabeling. For α-SMA staining, liver sections were incubated at 4 °C overnight with mouse anti-α-SMA monoclonal antibody (1:100) and thereafter with DAKO Envision's polymer of antibodies labeled with peroxidase for 1 h at room temperature. The DAB peroxidase substrate kit was used for its chromogenic substrate. Sirius red, which results in the red staining of all fibrillary collagen, was used to evaluate fibrosis. The liver sections were stained with 0.05% Fast-green FCF (ChemBlink, Inc., CAS 2353-45-9) and 0.05% Direct red 80 (Polysciences, Inc., CAS 2610-10-18) in saturated picric acid (Muto Pure Chemicals) for 90 min at room temperature. Positive area analyses were performed using the WinROOF image analysis software from 3 randomly selected fields among 3 mice for a total of 9 samples per group.

2.4. Measurement of hepatic hydroxyproline content

The hepatic hydroxyproline content was measured as described by Reddy et al. [15]. Briefly, approximately 40 mg of frozen liver tissue was hydrolyzed in 2 N NaOH for 10 min at 65 °C, followed by incubation at 120 °C for 20 min. The same amount of 6 N HCl was added and incubated at 120 °C for 20 min. Activated charcoal solution (10 mg/ml in 4 N KOH) and 2.2 M acetic acid–0.48 M citric acid buffer (pH 6.5) were added to adjust the pH to 7–8. After centrifugation, 100 mM chloramine T solution was added to the supernatant and incubated at room temperature for 25 min. After the addition of 1 M Ehrlich's solution (*p*-dimethylaminobenzaldehyde), samples were incubated at 65 °C for 20 min. Absorbance was measured at 560 nm. The hydroxyproline content is expressed in μg/mg of sample protein.

2.5. Isolation of HSCs and LSECs

Primary HSCs were isolated from the livers of male C57BL/6 mice by collagenase/pronase digestion and the Nycodenz gradient method as described previously [9]; the cells were then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Primary LSECs were isolated using a combination of rat anti-CD146 and sheep anti-rat IgG antibodies conjugated with magnetic beads from a fraction separated using the Nycodenz gradient method after the collagenase digestion of the livers of male C57BL/6 mice, according to the method described by Kitazume et al. [16]. The cells were then cultured in a DMEM/nutrient mixture F-12 (F12) containing 10% FBS.

2.6. Preparation of the LSEC conditioned medium (CM)

Briefly, 1×10^5 LSECs were seeded onto 6-well plates and precultured for 24 h with DMEM/F12 containing 10% FBS medium to grow the cells to confluency, followed by overnight starvation with DMEM/F12 containing 2% FBS at 37 °C. After the cells were rinsed with phosphate buffered saline (PBS), the medium was changed to 2 ml of DMEM/F12 containing 2% FBS and further cultured for 24 h to create LSEC CM.

2.7. Immunofluorescent staining

HSCs were fixed with 4% PFA for 10 min and incubated with 0.1% Triton X-100 in PBS for 20 min at room temperature. After blocking with 3% BSA in PBS for 40 min at room temperature, cells were incubated with FITC-conjugated anti-mouse α-SMA monoclonal antibody (1:200) for 2 h at room temperature. After being washed with PBS, the cells were mounted with Vectashield DAPI mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) and observed under a Zeiss LSM 700 laser scanning confocal microscope. The intensities of α-SMA and R58 LAP-DPs were calculated in each panel with ZEN software for quantitative fluorescence analyses.

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