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Adhesion of platelets through thromboxane A_2 receptor signaling facilitates liver repair during acute chemical-induced hepatotoxicity

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ABSTRACT

Aims: Platelets have been suggested to play an important role in liver regeneration and repair after hepatic resection and acute liver injury. However, the underlying mechanisms of liver repair remain elusive. Signaling through thromboxane prostanoid (TP) receptor participates in inflammation and tissue injury through platelet aggregation. On the other hand, TP receptor signaling also is involved in tissue repair and tumor growth through angiogenesis. The present study was examined whether or not TP receptor signaling contributes to liver repair and sinusoidal restoration from acute liver injury through platelet adhesion to the hepatic sinusoids.
Main methods: Carbon tetrachrolide (CCl_4) was used to induce acute liver injury in TP receptor knockout mice ($TP^{-/-}$ mice) and their wild-type littermates (WT mice).
Key findings: Compared with WT mice, $TP^{-/-}$ mice exhibited delayed in liver repair and sinusoidal restoration after CCl_4 treatment, which were associated with attenuated hepatic expression of pro-angiogenic factors. Intravital microscopic observation revealed that adhering platelets to the sinusoids was increased in WT livers during the repair phase as compared with $TP^{-/-}$ livers, and platelet adhesion was dependent on TP receptor signaling. The levels of hepatocyte growth factor (HGF) in platelets from WT mice treated with CCl_4 for 48 h were greater than those form $TP^{-/-}$ mice, and HGF enhanced the expression of angiogenic factors in cultured human umbilical vein endothelial cells (HUVECs).
Significance: These results suggested that TP receptor signaling facilitates liver repair and sinusoidal restoration from acute liver injury through HGF release from platelets adhering to the sinusoids.

1. Introduction

Platelets are small, specialized blood cells that are released as anuclear cytoplasmic bodies from megakaryocytes in the bone marrow. Although the main function of platelets is to halt hemorrhage after tissue trauma and vascular injury, platelets also participate in inflammation and tissue repair through paracrine pathways or direct cell–cell interactions [1]. The immediate appearance at the site of an injured vascular wall indicates that platelets may be an important trigger for angiogenesis and tissue regeneration [2]. In vitro studies have revealed that both platelets and platelet-microparticles stimulate endothelial cell proliferation and promote the formation of capillary-like structures [3]. Thrombocytopenia is associated with reduced angiogenic responses to inflammation and/or tissue injury [4]. Platelets appear to preferentially adhere to angiogenic blood vessels and interference with this adhesion process blunts blood vessel proliferation and induces hemorrhage from the angiogenic vessels [4]. The enhanced angiogenic response elicited by treatment with erythropoietin in ischemic tissue also appears to be dependent on platelet adhesion [5]. Upon activation, platelets secrete various growth factors and pro-angiogenic factors, which include vascular endothelial growth factor (VEGF) [6], stromal-derived factor-1 (SDF-1) [7], hepatocyte growth factor (HGF) [8], and others [9].

In the liver, accumulated evidence suggests that platelets also contribute to liver regeneration and repair after resection and injury [10]. Platelet-derived serotonin mediates the initiation of hepatocellular proliferation and liver regeneration after partial hepatectomy [11] and hepatic ischemia reperfusion [12]. Thrombocytosis is beneficial to liver regeneration after major resection of the liver [13]. In thrombocytopenic mice, regeneration after both ischemic injury and major tissue loss is severely impaired [14].

Thromboxane A_2 (TxA_2) is a potent stimulator of platelet activation, aggregation, and vascular constriction. It exerts its biological activity by binding to a G-protein-coupled-specific receptor, the thromboxane

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prostanoid (TP) receptor. The TxA_2 receptor, TP receptor was originally purified from human platelets, and was revealed to be a GPCR with seven transmembrane domains [15,16]. Accordingly, TP receptor signaling is essential for platelet shape change and aggregation. As the expression of TP receptors is localized in several smooth muscle types, TP receptor signaling is important in mediating vascular and bronchial smooth muscle contraction. TP receptors are also widely expressed in the cardiovascular system. In endothelial cells, TxA_2 accelerates the expression of adhesion proteins, leading to endothelial dysfunction [16,17]. Furthermore, TP receptors are abundantly expressed in lymphoid organs such as the thymus and spleen, indicating that TP receptor signaling plays a role in regulating immune responses [16].

TxA_2 is upregulated under pathological conditions such as ischemia and inflammation through interactions between blood elements, including platelets and microvascular endothelial cells in the liver [17,18]. During acute inflammation, TxA_2/TP receptor signaling pathway is involved in liver injury through enhancement of pro-inflammatory mediators [19] and regulation of tumor necrosis factor-alpha (TNF-α)-mediated hepatic microcirculatory dysfunction including leukocyte-endothelial interaction [20]. These studies indicate that TxA_2 plays a pro-inflammatory role in acute liver injury. However, we recently have revealed that another role of TP receptor signaling, demonstrating that TP receptor signaling facilitates liver repair after chemical-induced hepatotoxicity through the recruitment of macrophages into the injured liver [21]. TxA_2 may act as a potent angiogenesis stimulator directly as well as indirectly by supplying cytokines such as proangiogenic factors upon platelet aggregation [22,23].

Although platelets appeared to be essential for liver repair and regeneration after acute liver injury, it remains unknown whether or not TP signaling plays a role in liver repair through the accumulation of platelets into the liver during hepatotoxicity. Thus, the present study was conducted to examine if TP signaling has any effect on platelet recruitment to recover from liver damage during carbon tetrachloride (CCl_4)-induced acute liver injury.

2. Materials and methods

2.1. Animals

Male, 8-week-old C57BL/6J wild-type (WT) mice were purchased from CLEA Japan (Tokyo, Japan). Male TxA_2 receptor knockout mice ($TP^{-/-}$ mice, eight-week-old) were developed in our laboratory [24]. All animals were housed in an environmentally controlled room with a 12 h light/dark cycle and allowed free access to food and water. All animal experimental procedures were approved by the Animal Experimentation and Ethics Committee of the Kitasato University School of Medicine, and were performed in accordance with the guidelines for animal experiments set down by the Kitasato University School of Medicine.

2.2. Animal procedures

Mice were given a single dose of CCl_4 (1.0 ml/kg body weight; 1:4 (*v*/*v*) in olive oil; Sigma-Aldrich, St. Louis, MO) by intraperitoneal (i.p.) injection as reported previously [21]. In another set of experiments, some animals were treated with the TxA_2 synthase inhibitor (OKY-046, 50 mg/kg, i.p., Kissei Pharmaceutical Co. Ltd.), the TP antagonist (S-1452, 10 mg/kg, orally (p.o.), Shionogi Co. Ltd.) [20,21] or aspirin (0.1 mL / 10 mg of body mass, p.o., Merck) [25].

2.3. Experimental protocols

Animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) at 12, 48, 72, and 96 h after CCl_4 administration, and blood was drawn from the heart and then centrifuged. An automated analyzer was used to measure serum alanine aminotransferase (ALT) activity. Immediately after blood collection, the livers were excised and rinsed in saline, and a small section from each liver was placed in 4% paraformaldehyde.

2.4. Histology and immunohistochemistry

Excised liver tissues were fixed immediately with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for histological analysis [21,26]. Sections (4 μm thick) were prepared from paraffin-embedded tissue and subjected to hematoxylin and eosin (H&E) staining or immunostaining. To quantify the extent of necrosis, the percentage of necrosis was estimated by measuring the necrotic area relative to the entire histological section, and an analysis of the area was performed with a VH analyzer (Keyence, Japan). Sections were also stained for proliferating cell nuclear antigen (PCNA) (Invitrogen, Carlsbad, CA), and PCNA incorporation was measured. The number of PCNA-positive hepatocytes from 1000 hepatocytes was quantified in six separate high power fields (×400) for each animal. The percentage of PCNA-positive cells was calculated, and the results were expressed as a PCNA-labeling index.

2.5. Real-time RT-PCR analysis

Total RNA was homogenized from liver tissues and cultured cells in TRIzol reagent (Gibco-BRL/Life Technologies, Grand Island, NY), and single-stranded cDNA was generated from 1 μg of total RNA by reverse transcription using ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Quantitative PCR amplification was performed with SYBR Premix Ex Taq (Takara, Shiga, Japan). The gene-specific primers are shown in Table 1. Data were normalized to the level of GAPDH in each sample.

2.6. Cell culture

Human umbilical vascular endothelial cells (HUVECs) (Kurabo, Tokyo, Japan) were cultured in the medium, HuMedia-EG2 (Kurabo) [7,27]. HuMedia-EG2 media contain hydrocortisone, human epidermal growth factor, human fibroblastic growth factor, gentamycin, amphotericin, FBS, and heparin. The medium was then replaced with serum free-medium and the confluent HUVECs were treated with human HGF (R&D Systems, Minneapolis, MN) (100 ng/ml in PBS) for 6 h. The HUVECs were then harvested and homogenized in TRIzol (Gibco-BRL), and the levels of human VEGF, VEGFR1, VEGFR2, and CD31 mRNA were measured by real-time RT-PCR.

2.7. Platelet isolation and labeling

Anesthetized donor mice were bled via heart puncture into heparinized microtubes. Isolated platelets were labeled according to previously

Table 1
Primers for real-time RT-PCR.

Gene	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
Mouse		
VEGF	TCTTCTCATTCCTGCTTGTGG	GATCTGAGTGTGAGGGTCTGG
VEGFR1	CAAAGCCAGAGTCCTTCAGAG	TAGGAGAGCATTGGAAATTGG
VEGFR2	GGCTGAAAAGATTGGATCAGG	CCAGGAACAATGACACCAAGA
CD31	ACTTCTGAACTCCAACAGCGA	CCATGTTCTGGGGGTCTTTAT
TGF-β1	AACAATTCCTGGCGTTACCTT	TGTATTCCGTCTCCTTGGTTC
FGF^{-2}	CCTTGCTATGAAGGAAGATGG	TCCGTGACCGGTAAGTATTGT
α-SMA	AGGGAGTAATGGTTGGAATGG	TGATGATGCCGTGTTCTATCG
GAPDH	ACATCAAGAAGGTGGTGAAGC	AAGGTGGAAGAGTGGGAGTTG
Human		
VEGF	ATGAACTTTCTGCTGTCTTGG	ACCACTTCGTGATGATTCTGC
VEGFR1	TGGGAAACAGAATTGAGAGCA	GTTTCTTCCCACAGTCCCAAC
VEGFR2	CAGAGTTGGTGGAACATTTGG	AACAGGTGAGGTAGGCAGAGA
CD31	CTGAACCTGTCCTGCTCCATC	CCGACTTTGAGGCTATCTTGG
GAPDH	GAAGGTGAAGGACGGACTC	GAAGATGGTGATGGGATTTC

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