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Carbon tetrachloride-induced liver injury in mice is tissue factor dependent

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ARTICLE INFO

Article history:
Received 4 January 2015
Received in revised form
17 February 2015
Accepted 19 February 2015
Available online 26 February 2015

Keywords:
CCl_4
Tissue factor antisense
Oligonucleotide
Fibrin
Liver injury.

ABSTRACT

Tissue factor (TF) is a membranous glycoprotein that activates the coagulation system when blood vessels or tissues are damaged. TF was up-regulated in monocrotaline (MCT)/lipopolysaccharide (LPS) hepatotoxicity model. The present study aimed to test the hypothesis that TF-dependent fibrin deposition occurs in liver toxicity induced by CCl_4 in mice. Pericentral deposition of TF and fibrin is induced after CCl_4-induced liver toxicity. The toxicity was evaluated by determination of serum activities of ALT, AST and ALP as well as GSH content and histopathological changes. The results showed that injection of mice with TF-antisense deoxyoligonucleotide (TF-AS) prevented the accumulation of TF and fibrin in the hepatic tissues. Furthermore, it significantly restored blood biochemical parameters, GSH content and distorted histopathological features caused by CCl_4. The current study demonstrates that TF activation is associated with CCl_4-induced liver injury. Furthermore, administration of TF-AS successfully prevented this type of liver injury.

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1. Introduction

The liver plays several key roles in blood coagulation being involved in hemostasis. It is the site of synthesis of all coagulation factors and their inhibitors except for von Willebrand factor (vWf) (Rapaport, 2000). Among coagulation factors, tissue factor (TF), which is a transmembrane glycoprotein also known as factor III is the initiator of the coagulation cascade *in vivo* (Chu, 2005). TF plays an essential role in hemostasis by restraining hemorrhage after vessel wall injury. However, the emerging role of TF in the pathogenesis of certain diseases such as sepsis, atherosclerosis, certain cancers and diseases associated with fibrin deposition such as thrombosis, has focused consideration to the development of new inhibitors of TF in order to stop the progression of these diseases (Eilertsen and Østerud, 2004). TF is not normally present on unstimulated monocytes or endothelial cells, but it can be induced on these cells by stimulation of various physiological activation factors *in vitro* (Nakamura et al., 2002). TF initiates the extrinsic blood coagulation, which proceeds as Ca^{+2}-dependent extracellular signaling to activate factor VII to factor VIIa, which subsequently activates factor X (FX) to its active form (FXa) which in turn converts prothrombin (FII) into thrombin (FIIa). As a result, FIIa cleaves off fibrinogen (FBG) into fibrin monomers that cross-link to produce

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http://dx.doi.org/10.1016/j.etap.2015.02.012
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insoluble blood clots (Ten Cate et al., 1993). Activation of hepatic TF could contribute significantly to coagulation and tissue injury. Previous study reported activation of TF in MCT/LPS-induced liver injury in mice (Hammad et al., 2013). In addition, mouse hepatocytes TF mediate activation of coagulation during hepatocellular injury (Sullivan et al., 2012).

Several changes occur in acute liver injury as imbalance in hemostatic system that may lead to coagulation activation so accumulation and deposition of fibrin in the liver, thus altering normal blood flow (Weerasinghe et al., 2011). In addition, several studies showed that blocking of coagulation cascade by proteases inhibitors prevent liver injury (Kawasaki et al., 2014; Mikami et al., 2005). Although anticoagulants provide protection against the liver damage induced by many xenobiotics, they have disadvantages including bleeding (Copple et al., 2002; Pearson et al., 1996).

Antisense deoxyoligonucleotides (AS) are single-stranded deoxyoligonucleotides that complementarily binds to messenger RNA (mRNA). This binding results in the selective and catalytic degradation of the targeted mRNA leading to the reduction in target protein levels (Bennett and Swayze, 2010). Moreover, treatment of rats with TF-AS successfully reduced ischemia–reperfusion-induced liver injury (Nakamura et al., 2001). Yin et al. (2010) reported that *in vitro* treatment of human umbilical endothelial cells with TF-AS ameliorates anoxia–reoxygenation-induced injury (Yin et al., 2010).

Carbon tetrachloride (CCl_4) is used as industrial solvent and it is widely used as an animal model for investigation of xenobiotic-induced free radical-mediated hepatotoxicity (Rechnagel and Glende, 1973). A single dose of CCl_4 leads to centrilobular necrosis and steatosis (Pierce et al., 1987), while prolonged administration produces liver fibrosis, cirrhosis and hepatocellular cancer (HCC), (Perez Tamayo, 1983). An earlier study suggests that activation of the coagulation system occurs during CCl_4 hepatotoxicity (Duplantier et al., 2004) and its role in the pathogenesis is unclear. Using molecular intervention of TF-AS, we tested the hypothesis that CCl_4-induced liver injury is related to the activation of the coagulation system and whether this activation is TF dependent or not.

2. Materials and methods

2.1. *Animal model, experimental design and treatment protocol*

Male Swiss albino mice at 5 weeks of age and 20–25 g of weight upon injection were used. Animals were fed a standard chow and allowed access to water *ad libitum*. They were allowed to acclimate for 2 weeks in a 12-h light/dark cycle prior to use. Mice were randomly assigned into four groups (8 mice for each); vehicle treated group (mice received corn oil, 0.2 ml for each mice, i.p.), CCl_4 group (mice received CCl_4, i.p., in single dose of 1.5 ml/kg), scrambled TF (TF-SC) and TF-antisense (TF-AS) groups (mice received oligonucleotides (ODNs) in a single i.v dose of 6 mg/kg in 100 μl saline 3.5 h before CCl_4 injection) (Hammad et al., 2013). Twenty-four hour post-CCl_4 treatments, animals were euthanized under deep ether anesthesia for collection of blood and liver tissues. Samples were taken and processed by standard histological techniques, liver toxicity markers, H&E examination and immunofluorescence analysis. All procedures relating to animal care, treatments, and sampling were conducted in compliance with the guidelines of our institutional research ethical committee.

2.2. *Antibodies and chemicals*

Mouse monoclonal anti-TF antibody was obtained from Thermo Scientific Pierce (IL, USA). Polyclonal rabbit anti-human fibrinogen antibody was obtained from Dako (CA, USA). Goat anti-mouse Alexa fluor 488 was obtained from Invitrogen (TX, USA). Cy3-conjugated Goat anti-rabbit antibody was purchased from Jackson Immunoresearch (PA, USA). CCl_4 was purchased from Sigma–Aldrich (St. Louis, MO, USA). All antibodies were diluted and used according to the manufacturer's instructions.

2.3. *Tissue factor oligodeoxynucleotides*

The oligonucleotides (ODNs) used in the current study were purchased from integrated DNA technologies (CA, USA). The following sequences of the mouse TF antisense and scrambled nucleotides were used: TF-AS, 5′-CATGGGGATAGCCAT-3′; TF-SC, 5′-TGACGCAGAGTCGTA-3′. TF-SC was used as control.

2.4. *Blood sampling and processing*

Mice were anesthetized using jar containing diethylether (Halocarbon, River Edge, NJ, USA). Portion of retro orbital blood samples were collected by using heparinized microcapillaries (Optilab, Berlin, Germany) of each animal used for Complete blood count (CBC). Anthor portion was withdrawn and used for separation of serum to measure liver function parameters.

2.5. *Complete blood count (CBC)*

Blood cell counts were performed with VetScan HM2^{TM} Hematology analyser (Abaxis Inc., Union City, CA, USA).

2.6. *Biochemical and GSH analysis*

Serum enzymatic activities of transaminases (ALT and AST) and ALP were assessed colorimetrically according to the method of Reitman and Frankel (1957) and Belfield and Goldberg (1971), respectively. The liver homogenate was used for the determination of GSH content, according to Beutler et al. (1963) method.

2.7. *Processing of hepatic tissue samples and histopathology*

Liver specimens were fixed in Davidson's solution for 24 h and then transferred to 70% ethanol. Thereafter, the slides were dehydrated in gradient ethanol and cleared in xylene. The slides were embedded in paraffin blocks and sectioned in a thickness of 4 μm and stained with hematoxylin (RICCA Chemical Co., TX, USA) and eosin (EMD Chemicals, NJ, USA). For immunofluorescence, tissue samples were sectioned into 4 μm and fixed on positively charged slides.

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