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M1- and M2-macrophage polarization in rat liver cirrhosis induced by thioacetamide (TAA), focusing on Iba1 and galectin-3

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ABSTRACT

Introduction: Resident and exudate macrophages play an important role in the development of liver cirrhosis. Ionized calcium binding adaptor molecule 1^+ ($Iba1^+$) and galectin-3^+ ($Gal\text{-}3^+$) macrophages regulate liver fibrosis probably through pro-inflammatory and pro-fibrotic factors. Macrophages show polarized functions in liver fibrosis; however, M1-/M2-polarization of $Iba1^+$ and $Gal\text{-}3^+$ macrophages remains obscured. This study investigated the M1-/M2-polarized properties of $Iba1^+$ and $Gal\text{-}3^+$ macrophages in chemical-induced liver cirrhosis.
Materials and methods: Cirrhosis was induced in F344 rats by repeated injections of thioacetamide (100 mg/kg BW, twice a week for 25 weeks). Liver samples were collected from post-first-injection (PFI) week 5 to 25. Macrophage immunophenotypes and myofibroblasts in the fibrous bridges (FBs) and pseudolobules (PLs) were analyzed by immunohistochemistry. Expressions of M1- and M2-related factors were analyzed with RT-PCR, separately in FBs and PLs.
Results: Activation of myofibroblasts was most pronounced in livers at week 15. $CD68^+$ (M1), $CD204^+$ (M2), $Iba1^+$ and $Gal\text{-}3^+$ macrophages in the FBs increased gradually and peaked at week 15, consistent with the upregulation of both M1-(MCP-1, IFN-γ, IL-1β, IL-6, and TNF-α) and M2-(TGF-β1, IL-4, and IL-10) related factors. $Iba1^+$ and $Gal\text{-}3^+$ macrophages showed both M1- and M2-immunophenotypes. $CD163^+$ macrophages showed a persistent increase, consistent with TGF-β1 upregulation. MHC class II^+ macrophages increased in the developing fibrotic lesions, and then reduced in the advanced stage cirrhosis.
Conclusion: Both M1- and M2-macrophage polarizations occur during development of liver cirrhosis. $Iba1^+$ and $Gal\text{-}3^+$ macrophages participate in liver cirrhosis through production of both M1- and M2-related factors.

Introduction

Liver cirrhosis represents a common clinical endpoint of all chronic liver diseases and is characterized by advanced fibrosis and conversion of normal architecture into structurally abnormal pseudolobules. Patients with cirrhosis have a high risk of hepatocellular carcinoma or complications of portal hypertension (Friedman, 2008; George et al., 2009; Iredale et al., 2013). Various cells including hepatic macrophages and hepatic stellate cells (HSCs) have been found to interact in hepatic fibrosis and progression to cirrhosis (Holt et al., 2008).

Ontogenically macrophages may be derived from blood monocytes, resident tissue macrophages (Kupffer cells in the liver), and dendritic cells (Gordon and Taylor, 2005); these types differ in tissue distribution and functions (Takahashi et al., 1996). Recent cell surface phenotyping studies suggest that distinct macrophage phenotypes exist in the liver. CD68 is a glycoprotein on lysosomal membranes, particularly on the phagososmes in macrophages; its increased expression implies enhanced phagocytosis (Damoiseaux et al., 1994). The hemoglobin scavenger receptor, CD163 is expressed by tissue resident macrophages in normal condition and activated macrophages in pathological lesions; increased CD163 expression is associated with the phagocytosis and production of pro-inflammatory factors such as tumor necrosis factor-α (TNF-α) (Fabriek et al., 2005; Polfliet et al., 2006; Wójcik et al., 2012). CD204 molecule is expressed by Kupffer cells in the liver and involved in host defense through phagocytosis (Orr et al., 2011; Kiyanagi et al., 2011; Hara et al., 2013). MHC class II molecule is expressed on antigen presenting cells including dendritic cells and activated macrophages

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and plays a pivotal role in the interaction between macrophages and lymphocytes in the immune response (Otto et al., 2006; Kaneko et al., 2008; Conrad and Dittel, 2011).

Iba1 has been used to detect microglia in normal and pathological lesions in rats and mice (Ito et al., 1998, 2001); recently we found that Iba1 and Gal-3 participate in TAA-induced acute liver injury and repair through secretion of transforming growth factor-β1 (TGF-β1) and monocyte chemoattractant protein-1 (MCP-1) (Wijesundera et al., 2014). Iba1 may also play a role in inflammation such as migration, proliferation of macrophages, and signal transduction (Yang et al., 2005; Tian et al., 2006). Galectin-3 (Gal-3) is a member of the galectin family of mammalian proteins that binds to galactose-containing glycoconjugates in a Ca^{2+} independent manner and was originally described as an antigen expressed on the surface of activated macrophages (Barondes et al., 1994; Dong and Hughes, 1997). However, a very few information is available on properties of $Iba1^{+}$ and $Gal\text{-}3^{+}$ macrophages in chemical-induced cirrhosis.

Macrophages play an essential role in inflammation and host defense being an essential component of innate immunity (Gordon and Martinez, 2010). Similarly there are number of evidence that more than one type of macrophages appears in inflammation, suggestive of heterogeneity of macrophages; these macrophages can change their properties depending on microenvironmental conditions (Yamate et al., 2002; Mori et al., 2009). Macrophages have been classified as classically activated macrophages (M1) and alternatively activated macrophages (M2) (Stein et al., 1992; Duffield et al., 2005; Martinez et al., 2008). M1-macrophages are induced by interferon-gamma (IFN-γ) and liberate pro-inflammatory cytokines and function mainly in inflammatory reactions and tissue destruction (Martinez et al., 2008; Gratchev et al., 2006). In contrast, M2-macrophages are activated by interleukin-4 (IL-4) and produce anti-inflammatory/pro-fibrotic cytokines including IL-10 and TGF-β1 (Henderson et al., 2006; Martinez et al., 2008; Meghari et al., 2007); M2-macrophages resolve inflammatory responses and promote tissue remodeling and fibrosis (Gratchev et al., 2006; Sica and Mantovani, 2012). However, the molecular basis that mediates macrophage activities and polarization is still largely unclear.

Thioacetamide (TAA) is a well-known hepatotoxicant which causes centrilobular necrosis in the liver (Chilakapati et al., 2005; Fujisawa et al., 2011). Injected TAA is metabolized in the liver by flavin containing monozygenase and finally becomes TAA-S-oxide metabolite; the metabolite interferes with the motion of RNA from nucleus to the cytoplasm, resulting in cellular structural and functional deformation leading to membrane injury (Chen et al., 2004). Repeated injections of carbon tetrachloride (CCl_4) and TAA have been widely used in development of cirrhosis in rats (Ide et al., 2002; Muriel and Escobar, 2003); rat cirrhosis induced by TAA closely resembles human micronodular cirrhosis (Ide et al., 2002).

We set out to define the paradigm of M1-/M2-macrophage polarization in a rat cirrhosis model induced by repeated injections of TAA by macrophage immunohistochemistry and mRNA profiling for M1-/M2-related factors, which is the first attempt to define the macrophage polarization paradigm during progression of chemical-induced cirrhosis in rats.

Materials and method

Animals and experimental design

Twenty- one 6-week-old male F344 rats (110–120 g BW) were obtained from Charles River Japan (Hino, Shiga, Japan). They were housed in an animal room, maintained at 22 ± 3 °C with a 12- hour light-dark cycle. The animals were fed with a standard diet for rats (DC-8; CLEA, Tokyo, Japan) and provided with tap water *ad libitum*. Fifteen rats were given an injection of TAA (Wako Pure Chemical Industries, Osaka, Japan) dissolved in physiological saline (0.9% NaCl), twice a week at a dose of 100 mg/kg BW, intraperitoneally. Three rats were euthanized under isofluorane anesthesia on each of post-first-injection (PFI) weeks 5, 10, 15, 20, and 25. Control group received an equivalent volume of physiological saline intraperitoneally and three rats were euthanized each at weeks 5 and 25.

Experimental procedures were in agreement with our institutional guidelines on animal care and use, and were conducted in accordance with basic policies for the conduct of animal experimentation of the Ministry of Health, Labor and Welfare Standards relating to the Care and Management of Experimental Animals and the Act on Welfare and Management of Animals, Japan.

Histopathology

Liver tissues were removed and immediately fixed in 10% neutral buffered formalin (NBF) or in Zamboni's solution (0.21% picric acid and 2% paraformaldehyde in 130 mM phosphate buffer, pH 7.4). Tissues were also embedded immediately in Tissu Mount® (Chiba Medical Co, Saitama, Japan) and stored at −80 °C until use. Formalin- and Zamboni's solution-fixed tissues were embedded in paraffin and sectioned at 3–5 μm in thickness. Formalin-fixed, de-waxed sections were stained with hematoxyllin and eosin (HE) for histopathological observations and with the azan-Mallory stain for collagen deposition.

Single immunohistochemistry

Immunohistochemistry was performed with peroxidase-conjugated secondary antibody (Histofine simple stain MAX PO®; Nichirei Inc., Tokyo, Japan). Zamboni's solution-fixed sections were used. Information of primary antibodies used is shown in Table 1. Sections were incubated with 5% skimmed milk in phosphate buffered saline (PBS) for 30 min to inhibit nonspecific reactions. These sections were incubated overnight with a primary antibody at 4 °C. The sections were treated with 3% H_2O_2 in PBS for 30 min to quench the endogenous peroxidase activity and then followed by the application of secondary antibody for 30 min. Positive reactions were visualized with 3, 3′-diaminobenzidine (DAB; Vector Laboratories Inc., Burlingame, CA, USA). Sections were lightly counterstained with hematoxylin. For negative controls, tissue sections were treated with mouse or rabbit non-immune serum instead of the primary antibodies.

Double immunofluorescence

Fresh frozen sections (10 μm in thickness) were used for double immunofluorescence. Sections were fixed in a mixture of acetone:methanol (1:1) at 4 °C for 10 min followed by blocking with Image-iT® FX Signal Enhancer (Life Technologies, Eugene, OR, USA) for 30 min at room temperature and incubated with primary antibodies at 4 °C overnight. After rinsing with PBS, sections were incubated with goat anti-mouse IgG conjugated to Cy3® (Invitrogen, Carlsbad, California, USA; 1 in 1000) at room temperature for 30 min. The slides were mounted with SlowFade® Gold anti-fade reagent with 4′, 6-diamino-2-phenylindole (DAPI; Life Technologies, Eugene, OR, USA). Signals were analyzed with a confocal laser scanning microscope system (C1Si; Nikon, Tokyo, Japan).

Real-time PCR for M1- and M2-related factors

Real-time PCR was performed to evaluate the expression of M1- and M2-related factors. MCP-1, IFN-γ, IL-1β, IL-6, and TNF-α were examined as M1-related factors while TGF-β1, IL-4, and IL-10 were examined as M2-related factors. Liver samples were immediately soaked in RNA later® (Ambion, TX, USA) and stored at −80 °C. Real-time PCR was performed as described previously (Wijesundera et al., 2013). Briefly, total RNA was extracted from liver samples using an SV Total RNA isolation system (Promega, Madison, WI, USA). RNA was reverse-transcribed by using a Superscript VILO® reverse transcriptase (Invitrogen, Carlsbad, CA, USA). PCR was performed with SYBR green real-time PCR master