Biochemical and Biophysical Research Communications 418 (2012) 445–450

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Complement factor C5 deficiency significantly delays the progression of biliary fibrosis in bile duct-ligated mice

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ARTICLE INFO

Article history:
Received 4 January 2012
Available online 18 January 2012

Keywords:
MMP9
Leukocytes
Peribiliary infiltration
Cholestatic liver disease

ABSTRACT

Fibrogenesis represents the universal response of the liver to chronic liver injury. Complement factor C5 has been linked to fibrosis in murine toxic liver injury and human chronic hepatitis C. C5 may also play a central role in chronic cholestatic disorders, since the BA receptor FXR has been characterized as an activator of the *C3* gene. We aimed to investigate, whether C5 deficiency is able to prevent biliary fibrosis in the mouse bile-duct-ligation model.

BDL for 1–4 weeks was performed in either Hc^0/Hc^0 mice (deficient for C5) or WT controls. BA levels were measured by RIA. Histological examination included HE, sirius-red and immunohistochemistry. mRNA expression was quantified by RT-PCR. Protein expression levels were determined by Western blotting or ELISA. Enzymatic MMP-activity was analysed by zymography.

One week BDL leads to fibrosis in WT (F2.0 ± 0), while it is almost absent in Hc^0/Hc^0 mice (F0.5 ± 0.5). No differences in fibrosis can be detected at week-4. Together with delayed fibrogenesis at week-1, fibrotic markers are decreased in Hc^0/Hc^0 mice. Expression of the inflammatory cytokine TNF-α is decreased in Hc^0/Hc^0 mice. In parallel C5 deficiency leads to an attenuated peribiliary infiltration of $CD45^+$ cells in fibrotic areas together with decreased MMP-9 expression and gelatinase activity.

The present study proves a functional role of C5 during biliary fibrogenesis. C5 deficiency leads to attenuated inflammation and normalized MMP-9 activity concomitantly with a significant reduction of fibrosis. C5 appears to be an attractive target for future therapeutic intervention in chronic cholestatic liver disease.

1. Introduction

Cholestatic liver diseases account for a substantial subset of chronic liver disease in people and are among the leading indications for liver transplantation in all age-groups [1]. In adults, PBC, PSC and cholestatic forms of hepatitis frequently progress to cirrhosis and end-stage-liver disease [2,3]. Without treatment, most patients eventually develop fibrosis and cirrhosis of the liver and may need liver transplantation in the late stage of disease [4]. Although of different etiology, the consequence of impaired bile flow in all cholestatic disorders is the retention of bile constituents including BAs. Although, recent advances have been made in the pathophysiological understanding of liver fibrogenesis [5], the development of effective therapies for chronic cholestatic disorders is still impaired by our insufficient knowledge of the molecular mechanisms by which cholestasis/retention of BAs injures the liver, and which fibrotic mechanism is involved. In chronic cholestatic disorders, T-lymphocytes and cytokines mediate persistent bile duct damage and biliary cells secrete fibrogenic mediators activating extracellular matrix formation by neighboring portal

Abbreviations: α-SMA, alpha-smooth muscle actin; BA, bile acid; BDL, bile duct ligation; Col, collagen; FXR, farnesoid X receptor; HE, Hematoxilin&Eosin; Ntcp, Na-dependent taurocholate transporter; MMP, matrix metalloproteinase; Mrp, multidrug resistance associated protein; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; TGF, transforming growth factor; TNF, tumor necrosis factor.

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doi:10.1016/j.bbrc.2012.01.036

myofibroblasts [6,7]. In consequence, perisinusoidal hepatic stellate cells become activated and periportal fibrotic bands develop finally leading to end-stage cirrhosis. Irrespective of the well established cytoprotective and choleretic effects of the hydrophilic BA ursodeoxycholic acid (UDCA) no clear-cut benefit has been achieved with regard to disease progression and transplant-free survival in patients with PBC and PSC [8,9]. Recently, the side chain modified BA norUDCA has been shown to markedly improve biochemical and histological features in a mouse model of sclerosing cholangitis but clinical trials in humans are not available yet [10,11]. In the absence of an effective medical therapy, the delineation of further targets for intervention in cholestatic liver disease is urgently needed and represents the molecular basis for future treatment strategies.

It has been widely recognized that the complement system plays a critical role in the pathogenesis of a variety of chronic human disorders including chronic liver disease [12]. Activation of the complement system initiates a cascade resulting in the cleavage of the central molecule of the complement system, C3, which in turn leads to a downstream cleavage of C5. The resulting products C3a, C5a and other complement components are activators of distinct cell surface receptors translating risk signals into defined cellular responses [13].

More than two decades ago, it has been noticed that serum complement levels, particularly C3, are increased in patients with obstructive jaundice [14], PBC [15] and PSC [16]. More recent evidence suggests, that the increase of C3 in PBC patients is not due to the underlying disease entity but rather due to cholestasis in general [17]. Similar conclusions can be drawn from the description of a PSC patient with complete obstruction of the common bile duct whose serum complement C3 and C4 levels normalized upon surgical reconstitution of bile flow [18]. Intriguingly, long-term plasma exchange treatment of patients with PBC even improved liver function and decreased fibrosis marker procollagen III amino peptide along with reduced C3 activation [19].

These clinical observations highlight the possibility of a functional link between cholestasis or BA retention and complement activation on one side, and cholestasis-induced complement activation and fibrosis progression on the other side. Indeed, regulation of complement C3 expression by the nuclear BA receptor FXR has recently been demonstrated for both human and rodent genes [20]. Given the role of the complement system in chronic toxic liver injury in mice or chronic viral hepatitis C infection in humans [21,22], and the fact that different forms of liver injury activate fibrogenesis in a disease-specific fashion [23], we aimed to investigate the particular role of BA-induced complement activation in biliary fibrosis. Aim of this study was therefore to characterize the effects of C5-deficiency on fibrogenesis in the murine BDL model which exhibits similar structural alterations to those observed in chronic biliary obstruction in man.

2. Material and methods

2.1. Animals

Eight-week old C5-deficient (Hc^0/Hc^0) male mice (B10.D2-Hc0H2dH2-T18c/oSnJ) and age- and gender matched control animals (B10.D2-Hc1H2dH2-T18c/oSnJ) were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and kept under standard conditions. BDL or sham-laparatomy was performed in either C5-deficient mice ($n = 4$, each group) or WT controls ($n = 5$, each group) as described previously [24]. After 1 and 4 weeks the mice were sacrificed and liver tissue and blood samples were harvested. Paraffin-embedded sections from both time points were analyzed after HE and sirius-red staining for the degree of hepatic fibrosis. The staging was described previously [21]. Additionally liver fibrosis was assessed in all animals histologically by quantification of the sirius-red-positive area, using the Adobe Photoshop CS3 software. The animals received humane care and the study protocols were approved by the local Government's Animal Care Committee (Nr. 2008162).

2.2. mRNA isolation and real-time RT-PCR

Total RNA was isolated from liver by using RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. mRNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Mannheim, Germany). Expression was normalized against β-actin. Real-time-PCR was performed using ABI TaqMan probes on an Applied Biosystems 7900HT RT-PCR System. Taqman Probes: Ntcp: Mm00441421_m1, Mrp3: Mm00551550_m1, Mrp4: Mm01226381_m1, Col I: Mm00801666_g1, Col III: Mm01254476_m1, α-SMA: Mm00725412_s1, TGF-β: Mm03024053_m1, β6-Integrin: Mm00445326_m1, TNF-α: Mm99999068_m1, β-actin: 4352341E.

2.3. Western blotting

Similar amounts of serum proteins were separated by SDS–PAGE, transferred to PVDF membrane and probed with C3 antibody (Santa Cruz, SC 20137, USA). After incubation with species-specific HRP-conjugated secondary antibody (Dianova, Hamburg, Germany) immune complexes were detected using ECL-PLUS detection kit (GE-Healthcare, Freiburg, Germany). Densitometric quantification of Western blots was performed using Adobe Photoshop CS3.

2.4. MMP-9 Elisa

Tissue lysates were normalized for protein concentration and equal amounts were used for ELISA. Hepatic MMP-9 protein expression was quantified by ELISA according to the manufacturer's instructions (R&D Systems, DY909).

2.5. BA quantification

Serum BAs were measured using BA RIA Kit (MP Biomedicals, Ilkirch, France) according to the manufacturer's specifications.

2.6. Gelatinase assay

Proteolytic activity of the recombinant MMP-9 mutants was shown by gelatin zymography as described before [25]. For gelatin zymography 1 mg/mL gelatin was copolymerized in a 7.5% PAGE gel and 20 μg protein of pooled liver lysates of each group was loaded.

2.7. In situ zymography

An unfixed liver tissue cryoslice of 3 μm thickness was stained with 1 μM DAPI-solution for 3 min and subsequently washed in MMP-buffer. The fluorescein conjugated DQ gelatin (Molecular Probes) was dissolved at 99 °C in MMP-buffer containing 1% agarose. Using an applicator, a cover slip was coated on one side with the hot agarose buffer. After 30 s cooling at room temperature the cover slip is mounted onto the tissue slice and surplus buffer was rolled out to one side. The object slides were incubated overnight at 37 °C in a water saturated atmosphere. MMP-buffer: 50 mM TRIS pH7.4, 100 mM NaCl, 1 mM $CaCl_2$, 50 μM $ZnCl_2$.