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Increased activity of the complement system in the liver of patients with alcoholic hepatitis

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ABSTRACT

Inflammation has been suggested as a mechanism underlying the development of alcoholic hepatitis (AH). The activation of the complement system plays an important role in inflammation. Although it has been shown that ethanol-induced activation of the complement system contributes to the pathophysiology of ethanol-induced liver injury in mice, whether ethanol consumption activates the complement system in the human liver has not been investigated. Using antibodies against C1q, C3, and C5, the immunoreactivity of the complement system in patients with AH was examined by immunohistochemistry and quantified by morphometric image analysis. The immunoreactivity intensity of C1q, C3, and C5 in patients with AH was significantly higher than that seen in normal controls. Further, the gene expression of C1q, C3, and C5 was examined using real-time PCR. There were increases in the levels of C1q and C5, but not C3 mRNA in AH. Moreover, the immunoreactivity of C5a receptor (C5aR) also increased in AH. To explore the functional implication of the activation of the complement system in AH, we examined the colocalization of C5aR in Mallory–Denk bodies (MDBs) forming balloon hepatocytes. C5aR was focally overexpressed in the MDB forming cells. Collectively, our study suggests that alcohol consumption increases the activity of the complement system in the liver cells, which contributes to the inflammation-associated pathogenesis of AH.

1. Introduction

The liver plays a key role in innate immune defense against pathogens (for review, see Gao et al., 2008). The liver receives blood circulating from the intestines, which is rich in bacterial products, environmental toxins, and food antigens. The predominant innate immune system of the liver is comprised of phagocytic cells (such as neutrophils and macrophages), lymphocytic cells (natural killer and natural killer T), and humoral factors (complement factors and interferons). The complement system is a major component of the host innate immune system (for reviews see Gasque, 2004). The complement system can be activated by three different pathways: the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP). CP is activated by binding of a specific part of the antibody molecule (Fc) to the C1 component and involves C1q, C1r, C1s, C4, C2, and C3 components. LP recognizes pathogen-specific carbohydrate patterns by binding of the mannan-binding lectin (MBL) or ficolin to common carbohydrate structures on microorganisms. AP is continuously activated at very low levels through the spontaneous hydrolysis ("tickover") of the complement component C3 in plasma. All activation conditions result in the formation of C3 and C5 convertases and subsequent generation of major effector molecules such as C3a, C3b, and C5a. These effectors mediate direct lysis, via membrane attack complex (MAC) formation or immune adherence and phagocytosis of the pathogen (C3b and C4b), or function as chemokines, chemoattractants, and activators of immunocompetent cells (C3a and C5a).

While the primary source of plasma C1q remains obscure, hepatocytes are the primary cells responsible for biosynthesis of most of complement components found in plasma (Morgan and Gasque, 1997; Nagura et al., 1985; Qin and Gao, 2006). Hepatocytes are also primarily responsible for the biosynthesis of several complement regulator proteins found in plasma, such as factor I, factor H, and the C1 inhibitor (Morgan and Gasque, 1997). Immune cells and endothelial cells, which also biosynthesize complement components, have less contributions compared to hepatocytes (Morgan and Gasque, 1997). Although the complement system provides critical protection against invading organisms, accumulating evidence implicates activation of complements in chronic inflammatory diseases, such as alcoholic liver disease (ALD) (Gao et al., 2011; Pritchard et al., 2007; Qin and Gao, 2006; Wang et al., 2012) and non-alcoholic fatty liver disease (NAFLD) (Rensen et al., 2009). C3 and C5 have been implicated in liver fibrosis

Abbreviations: ALD, alcoholic liver disease; AH, alcoholic hepatitis; AP, alternative pathway; C1q, complement component 1 q; C3, complement component 3; C5, complement component 5; CP, the classical pathway; FFPE, formalin-fixed, paraffin-embedded; HCC, hepatocellular carcinoma; LP, lectin pathway; MDBs, Mallory–Denk bodies; NAFLD, non-alcoholic fatty liver disease.

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and regeneration. Hillebrandt et al. reported that C5 and C5aR play a critical role in the pathogenesis of liver fibrosis (Hillebrandt et al., 2005). C3 and C5 have been demonstrated to be involved in liver regeneration after partial hepatectomy or after toxic injury (Strey et al., 2003). There are increased mortality, parenchymal damage, and impaired liver regeneration after partial hepatectomy in C3- or C5-deficient mice (Strey et al., 2003).

Hepatic C1q and C3 deposition is markedly elevated in mouse ALD models (Cohen et al., 2010). Jarvelainen et al. reported that ethanol feeding to rats increased the deposition of complement proteins C3 and C8, but not C1, in the liver (Jarvelainen et al., 2002). Complement C3 has been shown to contribute to ethanol-induced liver steatosis in mice as mice lacking C3 (C3 −/−) did not develop hepatic steatosis or increased alanine aminotransferase (ALT) concentrations in response to acute or chronic ethanol exposure (Bykov et al., 2006). In the liver of ethanol-fed mice, ethanol activates CP through C1q binding to apoptotic cells. C1q deletion abolishes ethanol-induced complement activation and upregulation of TNFα and IL-6 expression in the liver and reduces ethanol-induced liver injury (Cohen et al., 2010).

Although these findings indicate that the activation of complement components contributes to inflammatory responses in ALD, the murine findings have not been studied in human. Rensen et al. reported that the complement system is activated in human NAFLD and its activation correlates with disease severity (Rensen et al., 2009). Moreover, despite the obvious ALD relevance of complement system and the wealth of animal data demonstrating its importance in experimental ALD models, little is known about its role in human ALD. There are fragmented data that are based on serum concentration of complements from human. It has been reported that acute alcoholic intoxication does not affect the concentration of total hemolytic complements (Spagnuolo and MacGregor, 1975). The level of circulating complement is increased in chronic alcoholics who withdrew from alcohol consumption after admission (MacGregor et al., 1978). However, the serum concentration of C3 is reduced in alcoholics with cirrhosis (Fox et al., 1971; Petz, 1971). To better understand the role of complements in ALD, our study examined the expression of the complement system in liver biopsies from patients with AH.

2. Materials and methods

2.1. Biopsies

Human archived formalin-fixed paraffin-embedded (FFPE) liver biopsies from patients who had AH (n = 3–5) were obtained from Harbor UCLA hospital archives. In all the cases MDBs were present in some hepatocytes of liver tissue sections from patients with AH but were not present in the normal control livers (control; n = 3). The biopsy sections were cut 4 μm thick.

2.2. Immunohistochemical staining

FFPE tissue slides were triple stained for various complements (Rabbit anti-C1q, Bioss, Woburn, MA; Rabbit anti-C3 and Rabbit anti-C5, Abcam, Cambridge, MA; Rabbit anti-C5aR, Novus Biologicals, San Diego, CA) and ubiquitin (Millipore, Temecula CA).

The complements were detected using the second antibody donkey anti-rabbit Alexa Fluor 488 (Jackson Lab, West Grove, PA). Ubiquitin was detected using the second antibody donkey anti-mouse Alexa Fluor 594 (Jackson Lab, West Grove, PA). All slides were stained with the nuclear stain DAPI (Molecular Probes, Eugene, OR). The slides were examined with a Nikon 400 fluorescent microscope. The fluorescence intensity of staining of the protein of interest was measured quantitatively using 40× objectives and a standard exposure time of 800 ms using a Nikon 400 fluorescent microscope with three filters (FITC, Texas red, and Tri-Color), and the Nikon morphometric system. The results were displayed as a graph attached to the immunofluorescent photography using a screen snip.

2.3. RNA isolation

For RNA isolation of FFPE tissue sections, paraffin was first removed from tissue samples when human liver biopsies were assayed. The paraffin-embedded tissue sections were mounted on a glass slide and dried at 60 °C for 30 min. The slides were then submerged in xylene at room temperature for 1 h changing the xylene once after 30 min. The samples were hydrated by washing progressively for 2 min in 100%, 70%, and 50% ethanol, and then pure RNase-free water before air-drying the samples on the slides for approximately 10 min. RNA isolation was processed using the Pinpoint™ Slide RNA isolation System II (ZYMO, Irvine, CA) by adding Pinpoint™ Solution directly to a small region of the tissue section to allow the solution to dry completely at the room temperature. The embedded tissue was then removed from the slide using a sterile blade or scalpel to scrape tissues from the slides followed by transferring the tissues to a micro-centrifuge tube for subsequent proteinase K digestion. The RNA was extracted and purified according to the manufacturer's protocol. DNA-free RNA can be obtained with subsequent DNase I treatment following the manufacturer's recommended protocol (ZYMO, Irvine, CA). The process described above was also followed for mice frozen liver tissues, except for the deparaffinization step. The quality and yield of the resulting total RNAs were assessed with an absorbance reading at 260 nm (A260) using a spectrophotometer (Thermo, Waltham, MA) by loading 1 μl of the extracted RNA.

2.4. Quantitative real-time PCR analysis

Synthesis of first-strand cDNAs was performed with the above mentioned total RNA (250 ng), and random hexamer primers using SuperSript III First-Strand Synthesis SuperMix (Invitrogen, San Diego, CA) following the instruction. Real-time PCR was performed using the Fast SYBR Green Master Mix on a StepOnePlus™ Real-time PCR System (Applied Biosystems) with a primer concentration of 200 nM. Primer sequences and the related gene Accession Number are listed in Table 1. Reaction conditions consisted of 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Single PCR product was confirmed with the heat dissociation protocol at the end of the PCR cycles. Human α-tubulin and mice β-actin were used as controls to normalize the starting quantity of RNA. Quantitative values were obtained from the threshold PCR cycle number (CT) at which point the increase in signal associated with an exponential growth for PCR product starts to be detected. The target mRNA abundance in each sample was normalized to its endogenous control level and the relative mRNA expression levels were analyzed using the ΔΔCT method. Reaction of each sample was performed in triplicate.

Table 1
Sequences of the forward and reverse primers of C1q, C3, and C5 for real-time PCR.

Gene name (species)	Accession number	Sequences of primer
C1q (Human)	NM_015991	Forward primer: 5′-CATCACCAACCAGGAA GAAC-3′ Reverse primer: 5′-TGTTGGTGGTGTCACA GAAG-3′
C3 (Human)	NM_000064	Forward primer: 5′-CCTGGCTCCACAGTTCTC TA-3′ Reverse primer: 5′-AGTGGAGAAGACCTGC TGTG-3′
C5 (Human)	NM_001735	Forward primer: 5′-ATTGGGAAGGCTACAC ATGA-3′ Reverse primer: 5′-TGCCTTGACAGTATCAGC AA-3′