Research Article

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C5aR, TNF-α, and FGL2 contribute to coagulation and complement activation in virus-induced fulminant hepatitis

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Background & Aims: Viral fulminant hepatitis (FH) is a disease with a high mortality rate. Activation of the complement system correlates with the development of FH. However, the key factors mediating complement activation in FH remain elusive.

Methods: Liver tissues were isolated from FH patients infected by hepatitis B virus (HBV) and from mice infected with murine hepatitis virus strain 3 (MHV-3). Wild type mice were treated with or without antagonists of C5aR or TNF-α, and mice deficient for *C5aR* (*C5aR*$^{-/-}$), *Fgl2* (*Fgl2*$^{-/-}$), and *Tnfα* (*Tnfα*$^{-/-}$) mice were not treated with the antagonists. C5b-9, C5aR, FGL2, CD31, CD11b, fibrin, TNF-α, and complement C3 cleavage products were detected by immunohistochemistry, immunofluorescence, or ELISA. Sorted liver sinusoidal endothelial cells (LSECs) or myeloid-derived (CD11b^{+}) cells were stimulated with C5a, TNF-α or MHV-3 *in vitro*. The mRNA expressions levels of *Fgl2* and *Tnfα* were determined by qRT-PCR analyses.

Results: We observed that complement activation, coagulation and pro-inflammatory cytokine production were upregulated in the HBV^{+} patients with FH. Similar observations were made in the murine FH models. Complement activation and coagulation were significantly reduced in MHV-3 infected mice in the absence of *C5aR*, *Tnfα* or *Fgl2*. The MHV-3 infected *C5aR*$^{-/-}$ mice exhibited reduced numbers of infiltrated inflammatory CD11b^{+} cells and a reduced expression of TNF-α and FGL2. Moreover, C5a administration stimulated TNF-α production by CD11b^{+} cells, which in turn promoted the expression of FGL2 in CD31^{+} LSEC-like cells *in vitro*. Administration of antagonists against C5aR or TNF-α ameliorated MHV-3-induced FH.

Conclusions: Our results demonstrate that C5aR, TNF-α, and FGL2 form an integral network that contributes to coagulation and complement activation, and suggest that those are potential therapeutic targets in viral FH intervention.

Keywords: C5a/C5aR; Fibrinogen-like protein 2 (FGL2)/fibroleukin; Complement; Fulminant hepatitis.

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Abbreviations: FH, fulminant hepatitis; HBV, hepatitis B virus; MHV-3, murine hepatitis virus strain 3; MAC, membrane attack complex; LSECs, liver sinusoidal endothelial cells; FGL2, fibrinogen-like protein 2; ALT, alanine aminotransferase; AST, aspartate aminotransferase; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; NPCs, non-parenchymal cells; RA, rheumatoid arthritis.

Introduction

Virus-induced fulminant hepatitis (FH) is a disease with a high mortality (>80%) in humans [1,2]. The clinical pathological characteristics of FH include hepatocellular necrosis, fibrin deposition, microvasculature thrombosis, and inflammatory cell infiltration [3,4]. Murine hepatitis virus strain 3 (MHV-3) induced FH is a prevalent model for investigating the underlying mechanisms of viral hepatitis, since MHV-3 infections in mice recapitulate the clinical symptoms of acute liver failure in FH patients [4–6]. Prothrombinase fibrinogen-like protein 2 (FGL2)/fibroleukin, which converts prothrombin to thrombin, is a key factor in fibrin deposition and microvascular thrombosis [7–11]. Large amounts of FGL2 have been detected in hepatocellular necrotic areas of the MHV-3-induced FH model, particularly in liver sinusoidal endothelial cells (LSECs) [10]. In *Fgl2*-deficient mice, fibrin deposition and survival following MHV-3 infection are significantly improved [4]. Hence, thrombosis induced by coagulation system activation plays a pivotal role in the pathogenesis of FH.

The activation of the coagulation pathway is accompanied by activation of the complement cascade during episodes of tissue injury and inflammation [12–15]. Recent studies have demonstrated that activation products of the coagulation cascade are capable of activating complement C3 and C5 [16–18], which are

two central components of the complement system. Additionally, signalling triggered by activated complement and subsequent responses in target cells are important for viral FH [19], which underscores the important function of the complement system in FH. However, the factors that control complement activation during FH have yet to be identified.

Complement activation is involved in many diseases, including infection [20,21], trauma [22], cancer [23], and autoimmune diseases [24]. All activation pathways (i.e., the classical, alternative, mannose-binding lectin and coagulation pathways) of the complement system cleave C3 into C3a and C3b fragments, which contribute to the formation of C5 convertase, and the subsequent cleavage of C5 into C5a and C5b fragments. C5b interacts with C6 to initiate the formation of the membrane attack complex (MAC, C5b-9), which clears pathogens via lysis or by inducing local tissue injury. C5a executes its biological actions by binding to its specific receptor C5aR (CD88), which is expressed on a variety of inflammatory cells [25], including monocytes, macrophages, and neutrophils.

In the present study, we sought to determine key factors that mediate complement activation in virus-induced FH. We found that the interaction of C5a and C5aR and the production of TNF–α and FGL2 were required for pathological complement activation during FH. Our results demonstrate that C5aR, TNF-α, and FGL2 form an integral network that contributes to coagulation and complement activation, and suggest that they are potential therapeutic targets in viral FH intervention.

Materials and methods

Clinical samples

FH patients with HBV infections were diagnosed in the Southwest Hospital (Chongqing, China) based on diagnostic criteria of acute liver failure established 2011 in Japan [26,27]. The medical history and results of physical and laboratory examinations of the FH patients are described in detail in Supplementary Table 1. Liver tissue samples were obtained from 6 patients with FH prior to liver transplantation. Control liver tissues were obtained from patients with benign hepatic haemangioma, who were proven to be free of HBV infection by laboratory investigations. Plasma samples were collected from 15 patients with FH and 15 healthy donors. The characteristics of these samples are described in Supplementary Tables 3 and 4. The sample collections were approved by the ethics committee of the Southwest Hospital (Chongqing, China) and all patients were informed about sample processing and signed the consent documents.

Virus

The MHV-3 virus was kindly provided by Prof. Qin Ning (Institute of Infectious Disease, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China). The detailed preparation, aliquoting and stocking of the virus have been described previously [28].

Mice and infections

C5aR$^{-/-}$ and *Tnfα*$^{-/-}$ mice were purchased from the Jackson laboratory. *Fgl2*$^{-/-}$ mice were kindly provided by Dr. Steve Smiley (The Trudeau Institute, NY, USA). C57BL/6 and BALB/c mice were obtained from the Animal Institute of the Academy of Medical Science (Beijing, China). The mice were backcrossed for at least nine generations onto a C57BL/6 background, and their homozygous wild type littermates were used as controls. The mice were kept under specific pathogen-free conditions at the Animal Center of the Third Military Medical University. All animal experiments were approved by the ethics committee of the Third Military Medical University.

Female mice (8–12 weeks) were intraperitoneally (i.p.) inoculated with 100 PFU of the MHV-3 virus, and survival was recorded daily. At indicated time points, the infected mice were sacrificed, and their livers and plasma were collected for immunohistochemistry (IHC), immunofluorescence, and enzyme-linked immunosorbent assay (ELISA) analyses. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using the Olympus AU5400 automatic biochemistry analyzer.

In some experiments, 10^6 U of TNF-α (Biolegend, CA, USA) were injected into C57BL/6 mice via the tail vein, and total protein was extracted from the liver for the determination of FGL2 and C3b+iC3b+C3c.

Isolation and analysis of intrahepatic leukocytes

Liver leukocytes were isolated as described previously [29–32]. Briefly, the livers from C57BL/6 mice with and without MHV-3 infection were perfused with 20 to 40 ml phosphate-buffer saline (PBS), minced with scissors and digested for 30 min with 4% collagenase (C7657, Sigma, St Louis, MO, USA) at 37 °C. The digested extracts were then pressed through 200 μm cell strainers to gain single-cell suspensions. A small aliquot was stained with CD45 (labelled with PE-Cy7) to assess the number of intrahepatic leukocytes. The remaining cells were subjected to density gradient centrifugation (with 25% and 50% percoll, and 1350 g for 30 min at 4 °C) for leukocyte isolation. Non-parenchymal cells (NPCs) were collected from the interface between the two density cushions of 25% and 50% percoll with a 10 ml pipette. The harvested cells were assessed for the percentages of $CD11b^{high}F40/80^{-}$ and $CD11b^{low}F4/80^{+}$ cells with flow cytometry after staining with CD11b-PE and F4/80-FITC antibodies (eBioscience). For the determination of intracellular TNF-α, cells were pretreated with a BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (555028; BD pharmingen; San Diego, CA, USA) and then stained with an APC-TNF-α antibody (MP6-XT22; 17-7321; eBioscience). The stained cells were then analysed on a BD Facscanto II cytometer.

Isolation and culture of the LSECs

For the isolation of LSECs, non-parenchymal cells from uninfected mouse livers were stained with CD146 as described previously [33], and $CD146^{+}$ cells were sorted with a BD SORP FACSAria II. Between 0.5 million and 2 million LSECs were obtained from each mouse with this method. The isolated LSECs were then cultured in 6 well plates coated with collagen, at a density of 5×10^5 /well in RPMI1640, containing 10% FBS, 10 ng/ml vascular endothelial growth factor (VEGF), and 10 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen, Carlsbad, CA).

Statistical analyses

GraphPad Prism 5 software was utilized for the statistical analyses. The statistical significances of the differences for mouse survival rates were analysed with ANOVA, while the others were analysed using Student's *t* test. p values <0.05 were considered to represent significant differences.

Supplementary Materials and methods

The immunohistochemical and immunofluorescent staining, ELISA, peritoneal residential macrophage isolation and stimulation, flow cytometry, and quantitative PCR protocols, as well as etanercept and C5aR antagonist treatments are described in the Supplementary Materials and Methods.

Results

Complement activation and coagulation during virus-induced FH

We first compared the difference in complement activation, coagulation, and inflammatory cytokine production between people with and without FH. We found that depositions of C3, C5b-9 and fibrin and the expression of FGL2 were significantly upregulated in FH patients (Fig. 1A). TNF-α, IFN-γ, IL-1β, and FGL2 levels were also significantly higher in the plasma of FH patients than in the plasma of healthy controls (Supplementary Fig. 1). Consistent with these results obtained from human subjects, C3 cleavage products (C3b+iC3b+C3c), C5b-9, fibrin and FGL2 were