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Complement mediated hepatocytes injury in a model of autoantibody induced hepatitis

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ABSTRACT

Despite multiple reports on autoantibody-initiated complement activation in autoimmune hepatitis (AIH), how does the humoral immunity contribute to the pathogenesis of AIH remained unclear. In this report, by adoptively transferring a polyclonal rabbit anti-OVA antibody into Hep-OVA Tg mice in which OVA is selectively expressed on the surface of hepatocytes, we found that excessive complement activation initiated by the autoantibody overwhelmed the protection of intrinsic cell surface complement regulators, and induced hepatocytes injury both *in vitro* and *in vivo*. The anti-OVA antibody induced hepatic injury in Hep-OVA Tg but not WT C57BL/6 mice as assessed by serum ALT levels and liver histopathology. Immunohistochemical analyses showed that after the antibody administration, there was massive complement activation on anti-OVA IgG coated hepatocytes in Hep-OVA Tg mice, but not in WT mice. Consistent with these results, depleting complement by cobra venom factor (CVF) prior to antibody injections protected Hep-OVA Tg mice from anti-OVA IgG induced hepatic injury. In addition, treating Hep-OVA Tg mice with recombinant mouse decay accelerating factor, a native complement inhibitor, protected them from autoantibody induced hepatitis. These results suggest that complement could play a pivotal role in liver specific autoantibody mediated hepatocyte injury in AIH, and that complement inhibitors could be, in principle, developed as novel therapeutics against AIH.

Introduction

Autoimmune hepatitis (AIH) causes continuing inflammation and necrosis, which progress to cirrhosis and eventually, liver failure (Czaja 2008a; Czaja 2009). Although circulating autoantibodies are hallmarks of AIH (Czaja 2009) and several antibodies against hepatocyte surface antigens have been identified in AIH patients (Lohr et al. 1992; Treichel et al. 1994; Yamauchi et al. 2004), whether autoantibodies are integrally involved in the pathogenesis of AIH, and if so, by which mechanism, have not been completely elucidated.

Complement is primarily produced by the liver and participates in many liver diseases [reviewed in Qin and Gao (2006)]. It can be activated through the classical pathway after the autoantibodies bind to their target antigens (Ferrone et al. 1973). Although self tissues are generally protected from autologous complement mediated injury by intrinsic cell surface complement regulators, i.e., decay accelerating factor (DAF) (Medof et al. 1984), CD46 (Seya et al. 1986) and CD59 (Zalman et al. 1987), excessive complement activation overwhelming the protection of these complement regulators can cause tissue damage. In fact, the antibody-initiated, complement mediated cytotoxicity has long been recognized to play a pivotal role in the pathogenesis of many autoimmune diseases in which autoantibodies are present (Sahu and Lambris 2000). However, despite several reported connections between complement activation and AIH (Yamauchi et al. 2005; Minuk et al. 2008; Bouron-Dal Soglio et al. 2008), the precise role of complement in autoantibody-induced liver injury in AIH remains elusive.

In this report, we systematically studied the distribution of intrinsic cell surface complement inhibitors on primary mouse hepatocytes, and the role of complement in a hepatitis model in which the liver injury was induced by administrating a polyclonal rabbit anti-chicken ovalbumin (OVA) IgG into the Hep-OVA Tg mice, which selectively express membrane bound chicken OVA protein

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on their hepatocytes and have been employed to study the cellular autoimmunity in AIH (Buxbaum et al. 2008). Our results indicate that complement activation is the primary mechanism underlying autoantibody-induced liver injury in this model, and complement inhibitors could be developed as new therapeutics for AIH management.

Methods and reagents

Mice and antibodies: Hep-OVA transgenic (Tg) mice on C57BL/6 (B6) background which selectively express membrane-bound OVA on hepatocytes were developed as previously described (Buxbaum et al. 2008). 8–12 w male Hep-OVA Tg mice and age matched B6 mice (Jackson Laboratory, ME) were used in all studies. Polyclonal rabbit anti-OVA antibodyI was purchased from Millipore (Billerica, MA), rat anti-mouse CD59 mAb (clone ER-MP20) was ordered from AbCam Inc. (Cambridge, MA) and rat anti-mouse Crry mAb (clone 1F2) was purchased from BD Biosciences (San Jose, CA). Rat anti-mouse C3 mAb (Clone RmC11H9) was ordered from Cedarlane Laboratories (Burlington, NC) and the rabbit anti-human C5b-9 antibody which cross-reacts with mouse C5b-9 was from Abcam Inc. (Cambridge, MA). Rat anti-mouse DAF mAb (clone 2C6) (Spiller et al. 1999) was kindly provided by Dr. BP Morgan (Cardiff University, U.K.). All studies were conducted using an approved Institutional Animal Care Protocol.

Primary hepatocyte isolation: the mouse liver was *in situ* perfused with 0.05% collagenase H (Roche Molecular Biochemicals, IN) via portal vein, and the primary hepatocytes were isolated as described before (Zhao et al. 2003). Cells were cultured in William's medium E with 10% fetal bovine serum (FBS) for 2 h and collected for analyses.

Complement mediated cytotoxicity assay: a BCECF-AM based complement mediated cytotoxicity assay was employed as described before (Li et al. 2009). Briefly, 2×10^5 freshly isolated primary hepatocytes were first loaded with 3 μM BCECF-AM (Invitrogen, CA) in MEM medium at 37 °C for 1 h. After washing, labeled hepatocytes were incubated at 37 °C with 50 μg/ml rabbit anti-OVA IgG and 30% mouse serum in 100 μl GVB/Ca^{2+} Mg^{2+} buffer (veronal-buffered saline supplemented with 0.1% gelatin, 5 mM $CaCl_2$ and 3 mM $MgCl_2$) for another 30 min. 1 mM EDTA was included to inhibit complement activation in the controls. Following incubation, complement mediated cell injury was assessed by measuring levels of converted BCECF released into the supernatants using a fluorescence microtiter plate reader (Molecular Devices, CA) with excitation and emission wavelengths of 485 and 538 nm. To calculate the percentage of BCECF release after complement mediated cellular injury, the following equation was used: percentage of BCECF release = $[(A-B)/(C-B)] \times 100\%$; where A represents the mean experimental BCECF release, B represents the mean spontaneous BCECF release and C represents the mean maximum BCECF released which was induced by incubating cells with 0.1% SDS. The cells were also collected and assessed for C3b deposition by staining with an anti-mouse C3 mAb followed by flow cytometry analysis as described before (Lin et al. 2001a).

Induction of autoimmune hepatitis: 0.5 mg of the rabbit anti-OVA IgG was injected into Hep-OVA Tg mouse through the tail vein. Livers and sera were collected 4 h later. Serum ALT levels were measured by an automatic biochemical analyzer in the Clinical Core Laboratory of University Hospital Case Medical Center, and livers were sectioned and analyzed by H&E staining and immunohistochemical staining.

Flow cytometry analysis and immunohistochemical staining: To examine the distribution of intrinsic cell surface complement regulators, 2×10^5 freshly isolated primary hepatocytes were incubated with 5 μg/ml of mAbs against mouse DAF, CD59 or Crry, respectively, or the same concentration of non-relevant rat IgG as negative controls. Mouse erythrocytes known to express all the three intrinsic cell surface complement regulators were included as positive controls. For immunohistochemical stainings, liver tissues were snap frozen in liquid nitrogen, then 7 μm cryosections were cut and stained with mAbs against rabbit IgG (rabbit anti-OVA IgG), mouse C3, mouse C5b-9 and mouse CD11b using a Vectastain ABC kit (Vector Labs, CA) following the manufacturer provided protocol. Non-relevant isotope IgGs were used as controls.

Complement depletion by CVF: to deplete complement, 20 μg of purified cobra venom factor (CVF) (Sigma, MO) was injected i.p. in each mouse. Serum samples were collected from the tail vein before and after CVF injection for standard E^{shA} C3b uptake assays (Lin et al. 2001b) to verify the depletion of complement. In brief, 5×10^5 E^{shA} were incubated at 37 °C with 10% of the serum samples collected before and after CVF injection in 100 μl GVB/Ca^{2+} Mg^{2+} buffer for 30 min, then stained with 5 μg/ml FITC labeled anti-mouse C3 mAb followed by flow cytometry analysis on a flow cytometer (LSR I, BD Biosciences, CA).

Recombinant soluble mouse DAF preparation and treatment: yeast *Pichia pastoris* expressing soluble mouse DAF CCP 1–4 with a C-terminus 6× his tag was developed in the lab (Lin et al. 2001b). For soluble mouse DAF preparation, recombinant yeast was cultured in YPD media and induced with 1% methanol for 2 d. Secreted soluble mouse DAF protein was purified from the supernatants by Ni^{2+} affinity chromatography (Qiagen, CA) and dialyzed against PBS. The concentrations of the resultant mouse DAF was measured using a Bio-Rad protein assay kit (Bio-Rad, CA) following the manufacturer provided protocol.

For DAF-based treatment, 200 μg of purified recombinant mouse DAF protein was injected i.p. per mouse 40 min before anti-OVA IgG administration. Sera and livers were collected 4 h after induction of hepatitis. Inhibition of systemic serum complement by administrated DAF protein was assessed by C3b uptake assays using antibody sensitized sheep erythrocytes (E^{shA}) as described above. Serum ALT levels and liver histopathology were assessed as described above.

Statistics: all experiments were repeated at least twice. Results were compared using the ANOVA test. A p value < 0.05 was considered significant.

Results

Mouse hepatocytes constitutively express DAF, CD59 and Crry.

To systematically examine the distribution of intrinsic cell surface complement inhibitors, i.e. DAF, CD59 and Crry on mouse hepatocytes, we isolated murine primary hepatocytes by collagenase digestion with high purify (>95%, data not shown) following an established protocol (Zhao et al. 2003). After the isolation, we stained the hepatocytes with respective mAbs followed by flow cytometry analysis. These assays showed that mouse primary hepatocytes constitutively express all intrinsic cell surface complement regulator DAF, CD59 and Crry (Fig. 1).

The anti-OVA antibody induces complement mediated hepatocytes injury *in vitro*.

We next tested whether the rabbit anti-OVA IgG activates complement and induces complement mediated hepatocyte injury *in vitro* using primary Hep-OVA Tg mouse hepatocytes by standard C3b uptake and BCECF-based cytotoxicity assays (Kolber et al. 1988). These experiments showed that Hep-OVA hepatocytes exhibited markedly increased C3b deposition after incubating with anti-OVA IgG and 30% normal mouse serum (Fig. 2(A)) compared to those with complement inactivation (1 mM EDTA). Consistent with the elevated C3b deposition, antibody sensitized Hep-OVA hepatocytes with complement activation exhibited significantly