Molecular Immunology 46 (2009) 1942–1950

Contents lists available at ScienceDirect

Molecular Immunology

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

Factor H facilitates the clearance of GBM bound iC3b by controlling C3 activation in fluid phase

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ARTICLE INFO

Article history:
Received 16 March 2009
Accepted 20 March 2009
Available online 2 May 2009

Keywords:
Dense deposit disease
Factor H-deficient mice
Factor H reconstitution
Restore C3 regulation in plasma

ABSTRACT

Dense deposit disease (DDD) is strongly associated with the uncontrolled activation of the complement alternative pathway. Factor H (CFH)-deficient ($Cfh^{-/-}$) mice spontaneously develop C3 deposition along the glomerular basement membrane (GBM) with subsequent development of glomerulonephritis with features of DDD, a lesion dependent on C3 activation. In order to understand the role of CFH in preventing renal damage associated with the dysregulation of the alternative pathway we administered purified mouse CFH (mCFH) to $Cfh^{-/-}$ mice. 24 h following the administration of mCFH we observed an increase in plasma C3 levels with presence of intact C3 in circulation showing that mCFH restored control of C3 activation in fluid phase. mCFH resulted in the reduction of iC3b deposition along the GBM. The exogenous mCFH was readily detectable in plasma but critically not in association with C3 along the GBM. Thus, the reduction in GBM C3 was dependent on the ability of mCFH to regulate C3 activation in plasma. Western blot analysis of glomeruli from $Cfh^{-/-}$ mice demonstrated the presence of iC3b. Our data show that the C3 along the GBM in $Cfh^{-/-}$ mice is the C3 fragment iC3b and that this is derived from plasma C3 activation. The implication is that successful therapy of DDD is likely to be achieved by therapies that inhibit C3 turnover in plasma.

1. Introduction

DDD is an inflammatory renal disease that is strongly associated with complement dysregulation. Around 50% of individuals with DDD will reach end-stage renal failure 10 years after the onset of the disease (Smith et al., 2007). DDD is characterized by electron-dense transformation of the glomerular basement membrane (GBM). The precise composition of the electron-dense areas is unknown but the margins of the deposits contain complement components C3, C5 and C9 in the absence of immunoglobulin (Sethi et al., 2009). DDD is strongly associated with dysregulation of the alternative pathway (AP) of complement activation and specifically with impairment of the function of the plasma protein complement factor H (CFH), the major physiological regulator of the AP. Moreover, DDD can also be triggered by the presence of C3 nephritic factor, an autoantibody that stabilizes the AP C3 convertase (an enzyme complex that cleaves C3), anti-factor H antibodies and dysfunctional C3 molecules (Linshaw et al., 1987; Marder et al., 1983; Meri et al., 1992). The enhanced AP activation in all these situations results in depletion of C3 in plasma.

Complement component C3 is a complex glycoprotein consisting of α and β chains containing a thioester bond responsible for its covalent binding to hydroxyl or amine groups present on target surfaces (Dodds et al., 1996; Sahu et al., 1994). C3 can assume four different conformational states governed by thioester bond rearrangement and release of small proteolytic fragments (Nishida et al., 2006). The generation of C3b, via proteolytic cleavage by C3 convertase, initiates a positive feed-back loop that amplifies the activation of the complement cascade. However, this system requires a strict regulatory mechanism to prevent host cell injury and to maintain the normal physiological functions of the complement system. Factor I (CFI) in the presence of its cofactors (CFH, MCP [membrane cofactor protein, CD46] or CR1 [complement receptor one, CD35]) inactivates C3b via cleavage of the C3 α-chain, releasing a 17-amino acid peptide termed C3f, forming iC3b. CFI also further degrades iC3b into C3c and C3dg using CR1 as a cofactor (Harrison and Lachmann, 1980).

Previously, we generated homozygous CFH-deficient mice ($Cfh^{-/-}$) enabling the contribution of CFH in the development of DDD to be investigated in an *in vivo* experimental model. $Cfh^{-/-}$ mice developed uncontrolled AP activation with reduced concentration of plasma C3 and presence of C3 breakdown products in circulation. In addition, they developed C3 deposition along the GBM and a glomerular lesion morphologically similar to human DDD (Pickering et al., 2002). Intercrossing $Cfh^{-/-}$ mice with mice

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doi:10.1016/j.molimm.2009.03.030

deficient in factor B prevented the development of DDD demonstrating that the spontaneous renal disease was dependent on C3 activation through the AP (Pickering et al., 2002). Intercrossing C*fh*$^{-/-}$ mice with mice deficient in CFI (*Cfi*$^{-/-}$) also prevented the development of DDD demonstrating that the spontaneous renal disease was dependent on the ability of factor I to cleave C3b to iC3b (Rose et al., 2008).

Current therapeutic approaches to DDD are aimed at slowing the progression of the renal damage by decreasing proteinuria, improving renal hemodynamics and limiting leukocyte infiltration in the kidney (Smith et al., 2007). DDD due to the generation and persistence of autoreactive antibodies (C3Nef and anti-CFH antibodies) would theoretically be amenable to treatments that inhibit the differentiation, maturation, and allostimulatory function of B and T lymphocytes. For individuals with CFH mutations, CFH replacement therapy would seem the most logical approach. In this respect it is notable that CFH-deficient patients with atypical haemolytic uremic syndrome have shown improvement in renal function following infusions of plasma (Nathanson et al., 2001).

Here, we investigated the effects of exogenous murine CFH (mCFH) on plasma and renal C3 regulation in C*fh*$^{-/-}$ mice. Administration of mCFH restored plasma C3 regulation in C*fh*$^{-/-}$ mice. It also altered the renal C3 staining pattern. Exogenous mCFH resulted in reduction in GBM C3 staining together with the appearance of mesangial and tubulo-interstitial C3 staining. The exogenous mCFH was readily detectable in plasma but could not be detected in association with C3 along the GBM. Thus, the reduction in GBM C3 was dependent on the ability to regulate C3 activation in plasma. Moreover, in this study we provide further evidence that iC3b is the fragment of C3 present along the GBM in C*fh*$^{-/-}$ mice.

2. Material and methods

2.1. Animals

C*fh*$^{-/-}$ mice were generated previously (Pickering et al., 2002). *CD11b*$^{-/-}$ mice were purchased from the Jackson laboratory. Mice deficient for both CFH and CD11b (C*fh*$^{-/-}$*CD11b*$^{-/-}$) were generated by inter-crossing C*fh*$^{-/-}$ and *CD11b*$^{-/-}$ mice. All experimental animals were age and sex-matched and were bred on to the C57BL/6 genetic background for at least 10 generations. All experimental procedures were done in accordance with institutional guidelines.

2.2. Purified mouse CFH (mCFH)

mCFH was purified from purchased whole serum (Innovative Research, MI, USA) using heparin affinity chromatography. Briefly, serum was treated with 7% polyethylene glycol 8000 (Sigma, Gillingham, UK) on ice. The pellet was dissolved in PBS, dialyzed against Tris–NaCl (Tris 20 mM; NaCl 50 mM pH 7.4) and applied to heparin affinity chromatography (Heparin Sepharose™ 6 Fast Flow, Amersham Pharmacia Biotech, Uppsala, Sweden). After extensive washing, proteins were eluted with a linear salt gradient (75–250 mM NaCl). CFH-containing fractions were pooled, dialyzed against Tris–HCl (20 mM, pH 8.6) and subjected to ion exchange chromatography (DEAE-sepharose™ Fast Flow, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The column was extensively washed and the bound proteins eluted with a linear salt gradient (0–300 mM NaCl). CFH-containing samples were pooled and dialyzed against PBS. After each chromatography step the samples were checked for the presence of CFH by western blot using polyclonal cross-reactive anti-human CFH (Quidel, CA, USA). The purity of the samples was assessed by Coomassie stained SDS-PAGE gels. Our preparations contained a major protein band at the expected molecular weight position for CFH. Purified CFH samples used in these experiments contained approximately 0.4 μg/mL of lipopolysaccharide (LPS) assayed using Limulus Amebocyte Lysate (LAL) QCL-1000 (Cambrex, MD, USA).

2.3. Tagging mCFH with Alexa Fluor 488

Purified mCFH was tagged with a fluorescein analogue dye, Alexa 488 by the interaction of tetrafluorophenyl ester moiety with primary amines present in CFH forming stable dye-protein conjugated according to manufacturer's instructions (Invitrogen, Paisley, UK). The efficiency of the tag was measured by spectrophotometry.

2.4. Administration of mCFH to C*fh*$^{-/-}$ mice

Animals were intraperitoneally (i/p) injected with 1 mg of purified mCFH preparation or an identical volume of phosphate-buffered saline (PBS). In view of the presence of LPS in the mCFH preparations groups of animals were also injected with purified LPS (*E. coli* O111:B4, Sigma–Aldrich Co., Gillingham, UK) to identify any LPS-dependent effects. Twenty-four hours after the injection mice were sacrificed and plasma and renal tissue collected.

2.5. Depletion of neutrophils in vivo

To achieve neutrophil depletion *in vivo* C*fh*$^{-/-}$ mice were injected i/p at day 0 with 0.5 mg of rat monoclonal IgG_{2b} anti-murine neutrophil antibody (Ly.6G, Santa Cruz Biotechnology, CA, USA). 24 h later mice received either mCFH (1 mg), LPS (0.75 μg) or PBS. Mice were sacrificed on day 2. Blood samples collected before and 24 h after administration of Ly.6G were analysed to confirm successful neutrophil depletion using FACS and peripheral blood film examination. Peripheral blood was collected in 5% EDTA and neutrophils were stained using Phycoerythrin-conjugated rat IgG_{2b} anti-mouse GR-1 (Euro Bioscience GmbH, Friesoythe, Germany) antibody in the presence of a saturating concentration of the monoclonal antibody 2.4G2 which blocks both FcγRII and III receptor sites. The results were analyzed with a FACS Calibur instrument (Becton Dickinson, CA, USA). Peripheral blood films were prepared using a drop of peripheral blood collected in EDTA and the neutrophils identified by their characteristic nuclear morphology.

2.6. Plasma mCFH detection

mCFH in the peripheral blood was detected by western blot using a cross-reactive polyclonal goat antibody against human CFH (Quidel, CA, USA).

2.7. Plasma C3 levels

C3 levels were measured by ELISA using goat anti-mouse C3 antibody (MP Biomedicals, OH, USA) as previously described (Pickering et al., 2007). Results were quantified by reference to a standard curve generated from acute phase sera containing a known quantity of C3 (Calbiochem, CA, USA).

2.8. Analysis of glomerular C3

Glomeruli were isolated from frozen sections using a laser capture microscope (Leica, Wetzlar, Germany) and dissolved in 10% SDS solution containing protease inhibitor cocktail (Sigma–Aldrich Co., Gillingham, UK). Solubilised dissected glomerular tissue was subjected to SDS-PAGE under reduced condition. C3 was then detected by western blot using a polyclonal goat antibody against mouse C3 (MP Biomedicals, CA, USA).