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Research paper

Recombinant factor VIII Fc (rFVIIIFc) fusion protein reduces immunogenicity and induces tolerance in hemophilia A mice

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ABSTRACT

Anti-factor VIII (FVIII) antibodies is a major complication of FVIII replacement therapy for hemophilia A. We investigated the immune response to recombinant human factor VIII Fc (rFVIIIFc) in comparison to BDD-rFVIII and full-length rFVIII (FL-rFVIII) in hemophilia A mice. Repeated administration of therapeutically relevant doses of rFVIIIFc in these mice resulted in significantly lower antibody responses to rFVIII compared to BDD-rFVIII and FL-rFVIII and reduced antibody production upon subsequent challenge with high doses of rFVIIIFc. The induction of a tolerogenic response by rFVIIIFc was associated with higher percentage of regulatory T-cells, a lower percentage of pro-inflammatory splenic T-cells, and up-regulation of tolerogenic cytokines and markers. Disruption of Fc interactions with either FcRn or Fcγ receptors diminished tolerance induction, suggesting the involvement of these pathways. These results indicate that rFVIIIFc reduces immunogenicity and imparts tolerance to rFVIII demonstrating that recombinant therapeutic proteins may be modified to influence immunogenicity and facilitate tolerance.

1. Introduction

Hemophilia A is an X-linked inherited bleeding disorder characterized by spontaneous and traumatic bleeding [1]. The pathophysiologic features of this disease are associated with very low levels or activity of factor VIII (FVIII) protein, arising because of genetic defects (e.g. intron 22 inversion, large deletions) [2]. Currently, the mainstay of treatment for hemophilia A is protein replacement therapy [3], one major complication of which is development of neutralizing antibodies, also known as inhibitors, to the infused FVIII. The incidence of inhibitor formation is estimated at 20–30% in all patients and at 30–40% in patients with severe disease.[4] The development of inhibitors results from a complex multifaceted immune response involving both genetic and environmental risk factors [5,6]. Several key molecules have been identified that correlate with inhibitor formation in patients with hemophilia. These include polymorphisms in the genes of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α), the anti-inflammatory cytokine interleukin-10 (IL-10), and the regulatory T cell (Treg) marker cytotoxic T-lymphocyte antigen-4 (CTLA-4). Higher levels of TNF-α and IL-10 have been demonstrated to correlate with higher incidence of inhibitors while higher CTLA-4 expression has been associated with a decreased incidence of inhibitors [7–9]. However, the presence of splenic IL-10 positive T-cells has also been associated with induction of FVIII tolerance in Hem A mice [10,11].

Interventions to mitigate rFVIII immunogenicity in experimental models have included impairing co-stimulatory signals during antigen presentation [12], inducing Tregs [13], presentation of FVIII antigen by immature dendritic cells [14], and designing FVIII molecules with fewer putative immunogenic epitopes. We therefore sought to investigate the immunogenicity and immune tolerance potential of recombinant FVIII Fc fusion protein (rFVIIIFc), which was recently approved as a long-acting FVIII replacement therapy for patients with hemophilia A. rFVIIIFc is composed of a single

Abbreviations: FVIII, factor VIII; rFVIIIFc, recombinant human factor VIII Fc; BDD, B-domain deleted; FL-rFVIII, full length recombinant factor VIII; Treg, regulatory T-cells; TNF-α, tumor necrosis factor-α; IFN-γ, Interferon-γ.

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molecule of B-domain deleted factor VIII fused to the Fc domain of human IgG1 [15,16]. The Fc portion enables the molecule to interact with the neonatal Fc receptor (FcRn), replicating the interaction that rescues IgG from lysosomal degradation pathways, resulting in a prolonged circulating half-life [17]. Immunomodulatory properties of Fc-containing fusion proteins have also been reported previously [18]. Of interest, two T-cell epitopes, termed Tregitopes, have been identified in the Fc region of IgG1 that are capable of activating Tregs [19,20].

In this report, we evaluated antibody and cellular immune responses to rFVIIIFc in hemophilia A mice and interrogated the pathways that potentially mediate rFVIIIFc immune tolerance. We also investigated receptor dependent mechanisms to delineate the possible downstream molecules that may promote the tolerogenic activity of rFVIIIFc.

2. Materials and methods

2.1. Mice

Hemophilia A (HemA) mice (C57BL/6) bearing a FVIII exon 16 knockout on a 129 × B6 background [21] were obtained from Dr. H. Kazazian (University of Pennsylvania). All animal procedures used were approved by the Institutional Animal Care and Use Committee and performed based on guidelines from the Guide to the Care and Use of Laboratory Animals.

2.2. Antibodies and reagents

Antibodies for FACS were obtained from BD Biosciences (Franklin Lakes, NJ) or eBioscience (San Diego, CA). Recombinant human B-domain-deleted FVIIIFc (rFVIIIFc), recombinant human B-domain-deleted FVIII (Biogen in-house produced) used in ELISA, rFVIIIFc IHH (amino acid substitutions I253A, H310A, H435A) and rFVIIIFc N297A (single amino acid substitution in the Fc domain) were produced as previously described [16]. Recombinant factor VIII products BDD-rFVIII Xyntha® (Wyeth Pharmaceuticals, Philadelphia, PA) and full-length FVIII Advate® (Baxter Healthcare Corporation, Westlake Village, CA) were purchased and reconstituted according to manufacturers' instructions.

2.3. Immunization/tolerance induction in mice

The study scheme for immunization and/or tolerance induction is depicted in Fig. 1A and B. Three treatment groups consisting of 8–10 week old male HemA mice received intravenous doses of 50, 100, or 250 IU/kg on days 0, 7, 14, 21, 35, and 53. Blood samples were collected by retro-orbital bleeding prior to dosing on days 0, 14, 21, 28 and 42. Plasmas were prepared, and anti-BDD-FVIII total binding and neutralizing antibody levels were determined using ELISA and Bethesda assay, respectively. Animals were euthanized on day 56 by CO_2 inhalation and spleens were dissected in sterile PBS to isolate single cell suspensions (Miltenyi Biotec, Cologne, Germany) and were either fixed in 3% formalin for FACS staining or stored in dissociation buffer for RNA isolation (Roche Applied Science, Indianapolis, IN). For immune tolerance studies, mice were first injected with 50 IU/kg on days 0, 7, 14, 21, and 35, followed by 250 IU/kg of rFVIIIFc once weekly for 4 weeks. Rechallenged animals were tested for anti-BDD-FVIII antibody levels in plasma collected on days 14, 21, and 28 post rechallenge. To test the immune response to non-specific antigens, mice were injected subcutaneously on days 42 and 49 with DNP-OVA at 100 μg per mouse in a 1:1 emulsion with Titermax Gold adjuvant from Sigma®. Antibody responses to DNP and OVA were measured using an anti-DNP Ig and anti-OVA Ig assay kit from Assay Diagnostics.

2.4. Anti-BDD-FVIII antibody ELISA

The standard used for mouse IgG was a polyclonal pool of anti-FVIII monoclonal antibodies prepared by mixing equal amount of GMA8002 (A1), GMA8008 (C2), GMA8011 (C1), GMA8015 (A2), GMA8016 (A2), GMA8005 (A1/A3) (Green Mountain Antibodies Inc, Burlington, VT; FVIII domain epitopes in parenthesis). Detection antibody used was goat anti-mouse IgG-HRP. Absorbance was measured on a Spectramax M2 plate reader (Molecular Devices).

2.5. Bethesda assay for determining neutralizing antibody titers

Plasma samples were mixed with known concentrations of BDD-rFVIII (in-house prepared) and incubated for 2 h at 37 °C. Residual FVIII activity in the mixture was then tested using a Coatest FVIII SP kit. The activity of FVIII was calculated against a standard curve generated with serially diluted BDD-rFVIII in naïve HemA mouse plasma.

2.6. FACS analysis

Splenic lymphocytes and dendritic cells were stained for surface and intracellular targets. For intracellular staining, cells were permeabilized with BD Fix-Perm solution (BD Biosciences) followed by incubation with respective antibodies in the same buffer. Fluorescence intensity was recorded using a BD FACS Canto II and analysis performed using FLOWJO software. For each sample 10,000 events were acquired on the flow cytometer. T-cells and dendritic cells were gated based on CD4+ and CD11c+ staining, respectively.

2.7. Real time PCR and real time PCR-based array analysis

Total RNA was isolated (Roche Applied Science, Indianapolis, IN) and reverse transcribed to cDNA (Qiagen, Hilden, Germany). PCR primers for the tested genes were designed and purchased from IDT technologies (Coralville, IA). SYBR green–based real-time PCR was carried out using Quantitect system (Qiagen, Hilden, Germany) or a PCR-based array for tolerance specific genes (PAMM047Z, T-cell Anergy and Immune Tolerance PCR Array; SA Biosciences, Frederick, MD) in an ABI 7900 Fast Block real-time PCR machine (Applied Biosystems, Foster City, CA). Results were analyzed using the 7500 software version 2.0.5 using the $2^{-\Delta Ct}$ relative quantification method [22], after normalization to GAPDH, HPRT, Hsp90ab, beta-actin, and GusB. mRNAs that displayed threshold cycles (Ct) >35 were excluded from the analysis.

2.8. T-cell proliferation and determination of interferon-γ (IFN-γ) levels

HemA mice (8–10 week old) were injected with rFVIII products once a week for 2 weeks. Seventy-two hours post the second injection mice were euthanized by CO_2 inhalation and splenic T-cells isolated using magnetic bead-based murine CD4+ T-cell isolation kit (Miltenyi Biotec, Germany). T-cells were then labeled with 10 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Life Technologies, Carlsbad, CA). Peritoneal macrophages were obtained from naïve HemA mice (8–10 weeks old) by euthanasia and peritoneal lavage with sterile PBS. Labeled T-cells from immunized mice were co-incubated with naïve peritoneal macrophages in the presence of BDD-rFVIII or vehicle or CD3/CD28 microbeads (positive control; Miltenyi Biotec) in X-VIVO 15 medium (Lonza) containing co-stimulatory antibodies namely anti-CD28 and anti-CD49d (BD Biosciences), for 96 h at 37 °C. IFNγ levels in the culture supernatant were measured using an ELISA kit from Meso Scale

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