Molecular Immunology 97 (2018) 1–7

Contents lists available at ScienceDirect

Molecular Immunology

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

Absence of a neutralizing antibody response to humanized cobra venom factor in mice

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ARTICLE INFO

Keywords:
Cobra venom factor
Humanized cobra venom factor
Complement
Complement depletion
Antibody response

ABSTRACT

Cobra venom factor (CVF) is the complement-activating protein in cobra venom. Humanized CVF (hCVF) is a human C3 derivative where the C-terminal 168 amino acid residues were replaced with the homologous sequence from CVF. hCVF has been shown in multiple models of disease with complement pathology to be a promising therapeutic agent, with no observed adverse effects. Here we describe the antibody response to hCVF in two different strains of mice. hCVF was able to repeatedly decomplement the mice after four injections in weekly intervals, demonstrating the absence of a neutralizing antibody response. In contrast, natural CVF caused decomplementation in all mice only after the first administration. After two additional administrations of natural CVF, decomplementation was inconsistent and varied tremendously from mouse to mouse. After the fourth administration, natural CVF was essentially unable to deplete complement, consistent with the known generation of a neutralizing antibody response. We also analyzed the IgG antibody response to hCVF. There was great variation, with approximately one quarter of the mice exhibiting non-detectable levels of anti-hCVF IgG, and another quarter very low levels. The levels of anti-hCVF IgG did not correlate with the levels of remaining C3. The anti-hCVF antibodies cross-reacted with natural CVF, recombinant CVF, and human C3. Whereas overall the level of anti-hCVF IgG cross-reacting with human C3 was lower compared to rCVF or nCVF, mice with higher levels of anti-hCVF IgG exhibited higher binding to CVF and human C3, excluding the possibility that higher antibody levels reflect preferential immunogenicity of CVF-specific or human C3-specific epitopes.

1. Introduction

Cobra venom factor (CVF) is the complement-activating protein in cobra venom. It is a structural and functional analog of complement component C3. When CVF is added to serum, it binds Factor B and leads to the formation of an alternative pathway C3/C5 convertase. The convertase (CVF,Bb) is physico-chemically stable, and resistant to inactivation by complement regulatory proteins Factors H and I, causing rapid cleavage of C3 and C5, and resulting in the depletion of the serum complement (reviews: Vogel and Fritzinger, 2010; Vogel and Fritzinger, 2017). The functional similarity of CVF to C3 is corroborated by its structure. Both proteins are synthesized as single-chain pro-proteins which are subsequently processed into the mature proteins with corresponding chain structures (Fig. 1). The sequence identity between CVF and mammalian C3 is about 50%, with a similarity of about 70% (Fritzinger et al., 1994). Both proteins exhibit a highly similar crystal structure with identical domains (Janssen et al., 2006, 2009; Krishnan et al., 2009).

Ever since it had been demonstrated that CVF can be safely administered to laboratory animals (Cochrane et al., 1970), the ability of CVF to exhaustively activate, and thereby deplete, complement has been exploited in innumerable studies as a tool to study the role of complement in host defense as well as pathogenesis of disease. As a matter of fact, our knowledge of the role of complement in the pathogenesis of many diseases was established by comparing normal animals with complement-depleted animals. As complement depletion by CVF eliminates the pathogenic contribution of complement to the disease process, complement depletion has more recently been recognized as a potentially valuable therapeutic approach in diseases with complement pathogenesis (Vogel and Fritzinger, 2007, Fritzinger et al., 2008; Vogel et al., 2014). However, CVF is immunogenic, generating an antibody response that limits its usefulness for complement depletion to a single

Abbreviations: CVF, cobra venom factor; nCVF, natural CVF as it occurs in cobra venom; rCVF, recombinantly produced CVF; hCVF, humanized CVF; huC3, human C3; muC3, murine C3

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https://doi.org/10.1016/j.molimm.2018.02.018
Received 1 October 2017; Received in revised form 30 January 2018; Accepted 25 February 2018

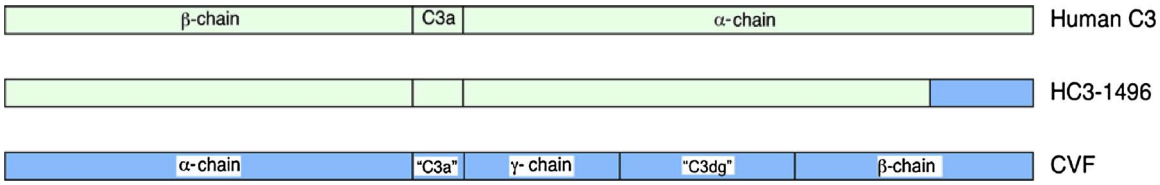

Fig. 1. Schematic representation of the chain structures of human C3, hCVF (HC3-1496), and CVF. Shown are the single-chain structures of pro-C3, pro-HC3-1496, and pro-CVF. The N-termini are to the left. The chain homologies are indicated. Please note that the nomenclature of the three chains of nCVF (α-chain, β-chain, γ-chain) was coined before their homology to C3 chains was established by N-terminal sequencing (Eggertsen et al., 1981), immunologic cross-reaction (Eggertsen et al., 1983), and molecular cloning (Fritzinger et al., 1994). Both hCVF and rCVF are usually expressed as a mixture of C3-like and C3b-like proteins (Fritzinger et al., 2009; Kock et al., 2004). Please note that rCVF is fully active without processing into the three-chain structure of nCVF (Kock et al., 2004).

injection (Cochrane et al., 1970, Pryjma and Humphrey, 1975). After identifying that the very C-terminal part of CVF harbors the important structures for forming a stable convertase (Grunwald et al., 1993; Hew et al., 2012), we attempted to overcome the limitation of immunogenicity by creating chimeric proteins in which C-terminal amino acid residues of human C3 were replaced by the homologous sequence from CVF. These chimeric proteins are human C3 derivatives, termed "humanized CVF" (hCVF), which exhibit rapid complement depletion in vitro and in vivo just like natural CVF (Fritzinger et al., 2009; Vogel and Fritzinger, 2010; Vogel et al., 2014). Our lead hCVF protein (termed HC3-1496) consists of human C3 in which the 168 C-terminal amino acid residues are homologous to CVF. HC3-1496 exhibits an overall protein sequence identity with human C3 of 94.3%; and even within the 168 C-terminal residues from CVF, 44.05% are identical to human C3 (Fig. 1).

hCVF has been shown in multiple preclinical disease models with complement pathogenesis to be a promising therapeutic agent. These include age-related macular degeneration (AMD) (Fritzinger et al., 2010), myocardial and gastrointestinal ischemia reperfusion injury (Gorsuch et al., 2009; Vogel et al., 2015), ventilator-induced lung injury (Takahashi et al., 2011), collagen-induced arthritis (Fritzinger et al., 2008), paroxysmal nocturnal hemoglobinuria (PNH) (Vogel and Fritzinger, 2010), myasthenia gravis (Vogel et al., 2014), and monoclonal antibody therapy of lymphoma (Wang et al., 2009). In three disease models (AMD, arthritis, and myasthenia gravis), multiple injections of hCVF resulted in depletion for up to 30 days, suggesting the absence of a neutralizing antibody response to hCVF. This observation was recently extended in a mouse model of hemophilia A, aimed at reducing the anti-Factor VIII immune response to treatment with recombinant Factor VIII in Factor VIII knock-out mice. A significant reduction in the levels of induced anti-Factor VIII antibodies was achieved by four cycles of complement depletion with hCVF in weekly intervals (Rayes et al., 2018). Here we report that repeated injections of hCVF into Factor VIII knock-out mice, as well as wild-type mice, lead to repeated complement depletion; and although antibodies are generated against hCVF, cross-reacting with natural CVF, recombinant CVF, and human C3, they do not neutralize the complement-depleting activity of hCVF.

2. Material and methods

2.1. Materials

Restriction enzymes and calf intestinal phosphatase were from New England Biolabs (Beverly, MA). T4 DNA ligase was either from New England Biolabs, or from Invitrogen (Carlsbad, CA). Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Fastart TAQ DNA polymerase and Fastart High Fidelity DNA polymerase were obtained from Roche Applied Sciences, Inc (Indianapolis, IN). The *D. melanogaster* S2 expression plasmid, pMT/Bip-V5-HisA, all *Drosophila* S2 media, and fetal calf serum were from Invitrogen. Human complement protein C3 was purchased from Merck Millipore (Merck Chemicals Ltd, Nottingham, United Kingdom). Natural CVF was purified from lyophilized *Naja kaouthia* venom (Miami Venom Laboratories, Punta Gorda, Florida, USA) as described (Vogel and Müller-Eberhard, 1984). Recombinant CVF (rCVF) was produced in *D. melanogaster* S2 cells as described (Kock et al., 2004). rCVF is a mixture of C3-like and C3b-like proteins (Kock et al., 2004) (Fig. 1). Polyclonal goat anti-mouse C3 antibodies were purchased from MP Biomedicals (Illkirch, France). Biotinylated polyclonal goat anti-mouse C3 antibodies and streptavidin-conjugated horseradish peroxidase (HRP) were from R&D systems (Lille, France). HRP-coupled polyclonal goat anti-mouse IgG antibody was from Southern Biotech (Anaheim, CA).

2.2. Preparation of the plasmid expressing hCVF protein HC3-1496

This plasmid was prepared in a manner very similar to the method described earlier for the preparation of the plasmid expressing hCVF protein HC3-1348 (pMB-HC3-1348) (Fritzinger et al., 2009). Initially, two PCR reactions were performed to obtain the human C3 and CVF portions of the coding sequence, the first using pBS-HuC3(2) as a template and HuC3H5-3-F1 (TCTGTGTGGCAGACCCCTTCGAGG) and HuC3H5-4-R1(2) (GAGAAGGCCTGTTCCTTTATCCGGATGGTAGAACC GGGTAC) as primers, and the second using pCVF-FL3Δ as a template and HuC3H5-4-F2(2) (CCGGTTCTACCATCCGGATAAAGGAACAGGCC TTC) and HuC3H5-3-R2 (CATCCATGACATAGATATCATTACCATCTTG) as primers. Following the PCR reaction, the products were purified using the Qiagen PCR purification kit, and combined in an overlap extension PCR reaction, using the two PCR fragments as templates and HuC3H5-3-F1 and HuC3H5-3-R2 as primers. This PCR product was purified, cut with BstBI and gel purified as described above. It was then ligated into pHC3-1550(-sig) (Fritzinger et al., 2009) that had been BstBI cut and dephosphorylated with calf intestinal phosphatase. Orientation of the inserts was determined by EcoRI digestion, and clones with the inserts in the correct orientation were sequenced to ascertain the correct sequence with a lack of PCR-induced mutations. The resulting plasmid was called pHC3-1496. The HC3-1496 coding sequence was amplified by PCR. There were two amplifications, one to produce a fragment coding for HC3-1496 with the native human C3 signal sequence, and one coding for HC3-1496 with a mouse IgG signal sequence. For producing the coding sequence with the native C3 signal, the following primers were used: PNAtf: 5'-gcaagcttGCCGCCACCATG GGACCCACCTCAGGTC-3' and Pnatr: 5'-ccgcggccgcTTAAGTAGGGCAG CCAAACTCAGTCAAT-3'. The primers used for producing the HC3-1496 with the mouse IgG signal sequence were Pmsp: 5'-gcaagcttGCCGCCA CCATGGAGACCGACACACTGCTGCTGTGGGTGCT GCTGCTGTGGGTC CCCGGCTCCACTGGAAGTCCCATGTACTCTATCATCACC CCCAAC-3' and Pnatr: 5'-ccgcggccgcTTAAGTAGGGCAGCCAAACTCAGTCAAT-3′. The resulting PCR products were gel purified and isolated from the gel using the Qiagen QIAquick Gel Extraction Kit, and cut with HindIII and NotI. The fragments were then cloned into pOptiVEC-TOPO that had been cut with the same enzymes. The expression vectors were called pOptiVEC-1496(sig)-3# and pOptiVEC-1496-3# respectively.