Molecular Immunology 67 (2015) 407–415

Contents lists available at ScienceDirect

Molecular Immunology

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

Insights into the effector functions of human IgG3 in the context of an antibody targeting transferrin receptor 1

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ARTICLE INFO

Article history:
Received 14 December 2014
Received in revised form 19 June 2015
Accepted 1 July 2015
Available online 29 July 2015

Keywords:
Antibodies
Effector functions
ADCC
CDC
Human IgG3
TfR1

ABSTRACT

The transferrin receptor 1 (TfR1) is involved in cellular iron uptake and regulation of cell proliferation. The increased expression of TfR1 observed in malignant cells, compared to normal cells, together with its extracellular accessibility, make this receptor an attractive target for antibody-mediated cancer therapy. We have developed a mouse/human chimeric IgG3 specific for human TfR1 (ch128.1), which shows anti-tumor activity against certain malignant B cells *in vitro* through TfR1 degradation and iron deprivation, and *in vivo* through a mechanism yet to be defined. To further explore potential mechanisms of action of ch128.1, we examined its ability to induce antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-mediated cytotoxicity (CDC). We now report that ch128.1 is capable of mediating ADCC and CDC against malignant B cells, which is consistent with its ability to bind FcγRI, FcγRIIIa, and the complement component C1q. To delineate the residues involved in these effector functions, we developed a panel of three constructs with mutations in the lower hinge region and C_H2 domain: 1) L234A/L235A, 2) P331S, and 3) L234A/L235A/P331S. The triple mutant consistently displayed a significant reduction in ADCC, while the L234A/L235A mutant exhibited less reduction in ADCC, and the P331S mutant did not show reduced ADCC. However, all three mutants exhibited impaired binding to FcγRI and FcγRIIIa. These results suggest that all three residues contribute to ADCC, although to different degrees. The P331S mutant showed drastically decreased C1q binding and abolished CDC, confirming the critical role of this residue in complement activation, while the other residues play a less important role in CDC. Our study provides insights into the effector functions of human IgG3 in the context of an antibody targeting TfR1.

1. Introduction

TfR1, also known as CD71, is a type II transmembrane homodimeric protein involved in cellular iron uptake and regulation of cell proliferation (Daniels et al., 2012a, 2006a,b). It is constitutively internalized and transported back to the cell surface. Cellular uptake of iron occurs through its interaction with Tf, which is internalized via receptor-mediated endocytosis. This iron-Tf/TfR1 complex is delivered into endosomes, where the decrease in pH facilitates the release of iron, which is then transported out of the endosomes into the cytosol (Daniels et al., 2006a). Increased expression of TfR1 has been observed in a variety of malignant cells compared to normal cells, and its expression correlates to tumor

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cell-mediated phagocytosis; AP, alkaline phosphatase; CDC, complement-mediated cytotoxicity; C_H2, constant domain 2; DNP, 2,4-dinitrophenyl; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FcγR, Fc gamma receptor; FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetate; NK, natural killer cells; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; PMN, polymorphonuclear leukocytes; Tf, transferrin; TfR1, transferrin receptor 1.

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http://dx.doi.org/10.1016/j.molimm.2015.07.001
0161-5890/

grade or prognosis in certain malignancies (Daniels et al., 2006a). Together, the properties of TfR1 make it an attractive target for cancer therapy. One strategy of targeting the TfR1 for cancer therapy is to antagonize its function using monoclonal antibodies to block iron uptake via inhibiting the binding of Tf to its receptor, blocking TfR1 internalization, or inducing TfR1 degradation. All these mechanisms ultimately lead to cytotoxicity through iron starvation. Another strategy exploits the TfR1-mediated endocytosis to deliver therapeutic agents into the cytoplasm of malignant cells. This can be accomplished by conjugating the therapeutic agent to Tf or to antibodies targeting the TfR1 (Daniels et al., 2006b, 2012a; Tortorella and Karagiannis, 2014).

In order to target the TfR1 as a potential cancer therapy, we developed a mouse/human chimeric IgG3 specific for the human TfR1 (ch128.1), previously known as anti-hTfR IgG3, as well as a derivative that has avidin genetically fused to the carboxy-terminus of the heavy chain (ch128.1Av), previously known as anti-hTfR IgG3-Av, to serve as a universal vector for delivery of biotinylated therapeutic agents into cancer cells (Ng et al., 2002, 2006; Rodriguez et al., 2007). Neither ch128.1 nor ch128.1Av inhibits Tf binding to TfR1 and are internalized through binding to TfR1. Importantly, both antibodies exhibit direct anti-proliferative/pro-apoptotic activity against a variety of malignant hematopoietic cells, including malignant B cells, *in vitro* through the induction of TfR1 degradation and subsequent lethal iron deprivation (Ng et al., 2006; Ortiz-Sanchez et al., 2009; Rodriguez et al., 2011). Interestingly, fusion of avidin to the antibody ch128.1 results in enhanced TfR1 degradation and cytotoxicity *in vitro* (Daniels et al., 2011; Ng et al., 2002, 2006; Rodriguez et al., 2011). However, the levels of sensitivity to ch128.1 and ch128.1Av vary among cell lines (Daniels et al., 2007, 2011; Ng et al., 2006; Ortiz-Sanchez et al., 2009). As originally designed, ch128.1Av has been successfully used to deliver biotinylated toxin saporin, a ribosome inactivating protein (Daniels et al., 2007; Daniels-Wells et al., 2013), and lentivirus for gene therapy (Leoh et al., 2014; Morizono et al., 2009). Importantly, significant anti-tumor protection against xenograft models of the human B-cell malignancy multiple myeloma in SCID-Beige mice was observed using ch128.1 or ch128.1Av alone (Daniels et al., 2011).

Although ch128.1Av exhibits superior cytotoxic activity *in vitro*, ch128.1 confers stronger anti-tumor protection *in vivo,* even against cell lines that showed low or no sensitivity to this antibody *in vitro* (Daniels et al., 2011). This could be due to the lower bioavailability of the avidin fusion protein, since avidin has been shown to accumulate in the liver and is rapidly cleared from the circulation (Rosebrough and Hartley, 1996). The mechanism of the *in vivo* protection conferred by ch128.1 and ch128.1Av remains undefined and may occur through multiple non-exclusive pathways.

The *in vivo* protection against tumor growth mediated by ch128.1 could be due to iron starvation resulting from TfR1 degradation, as previously observed *in vitro* (Ng et al., 2006), an effect that might be enhanced *in vivo,* increasing the sensitivity of malignant cells to the antibody. The antibody may also potentially interfere with receptor internalization through the binding to TfR1 on cancer cells and FcγRs on the surface of immune cells in the tumor microenvironment, which would also result in iron deprivation and cancer cell death. Alternatively and non-exclusively, ch128.1 may induce cell death through eliciting antibody Fc effector functions such as ADCC, ADCP, and CDC, as observed with other antibody therapies (Bakema and van Egmond, 2014; Meyer et al., 2014; Nimmerjahn and Ravetch, 2007, 2008). ADCC can be mediated through activation of a variety of FcγR-bearing effector cells such as NK cells, monocytes/macrophages, dendritic cells, and PMN such as neutrophils (Clynes et al., 2000; Hernandez-Ilizaliturri et al., 2003; Hubert et al., 2011; Pincetic et al., 2014; Schmitz et al., 2002), while complement activation is triggered via binding of C1q, the recognition component of the initializing complex in the classical complement cascade (Meyer et al., 2014). Importantly, ADCC has been described as a major mechanism of action for tumor-targeting antibodies (Nimmerjahn and Ravetch, 2007). Antibodies targeting malignant B cells, such as rituximab, are capable of inducing both ADCC and CDC (Amoroso et al., 2011; Cardarelli et al., 2002; Rose et al., 2002). Since the *in vivo* model we employed (SCID-Beige) lacks functional NK cells (Daniels et al., 2011), the anti-tumor activity conferred by ch128.1 may be mediated, at least in part, by other effector cells such as monocytes/macrophages.

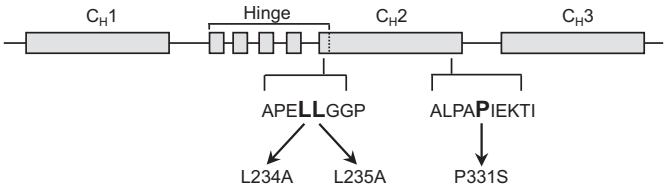

Fig. 1. Development of ch128.1 mutants. Schematic representation of the DNA encoding the heavy chain of human IgG3. Based on previous reports showing residues involved in FcγRI, FcγRIIIa, and C1q binding, the following ch128.1 mutants were developed: L234A/L235A/P331S, L234A/L235A, and P331S. This drawing is not to scale.

The objective of the present study is to explore the ability of ch128.1 to mediate antibody effector functions. We now report, for the first time, that ch128.1 is capable of eliciting ADCC and CDC against malignant B cells. We also address the contributions of the amino acid residues L234, L235, and P331 of the heavy chain of human IgG3 in these antibody effector functions in the context of TfR1 targeting in malignant B cells.

2. Materials and methods

2.1. Cell lines

The B-cell non-Hodgkin lymphoma cell line Ramos (human Burkitt lymphoma, American) was obtained from American Type Culture Collection (Manassas, VA) and J774.2, a mouse macrophage-like cell line, was a kind gift from Dr. Sherie L. Morrison (University of California, Los Angeles, CA). Both cells lines were grown in RPMI 1640 (Life Technologies, Inc., Carlsbad, CA) containing 10% heat-inactivated FBS (Atlanta Biologicals, Atlanta, GA) and penicillin/streptomycin in 5% CO_2 at 37 °C. PBMC from healthy volunteers were obtained from the UCLA Center for AIDS Research Virology Core Laboratory.

2.2. Construction of ch128.1 mutants

ch128.1 contains the variable regions of the murine monoclonal anti-human TfR1 IgG1 128.1 (Ng et al., 2006). Based on previous reports, mutations were designed to disrupt binding to FcγRI and FcγRIIIa (L234A/L235A), C1q (P331S), or all three (L234A/L235A/P331S), as depicted in Fig. 1 (Canfield and Morrison, 1991; Hezareh et al., 2001; Idusogie et al., 2000; Oganesyan et al., 2008; Tao et al., 1993). These mutations were generated in the γ3 heavy chain expression vector by GenScript USA, Inc. (Piscataway, NJ). NS0/1 murine myeloma cells were transfected with the human γ3 heavy and human κ light chain expression vectors to express the ch128.1 mutants as previously reported for wild type ch128.1 (Ng et al., 2006). Cells expressing the ch128.1 mutants and wild type ch128.1 were grown in roller bottles and antibodies were purified from cell culture supernatants using affinity chromatography as previously described (Helguera and Penichet, 2005; Leoh et al., 2014; Ng et al., 2006). SDS-PAGE analysis under reducing and non-reducing conditions was performed to confirm the molecular weight and assembly of the mutant antibodies.