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Research paper

Humanised recombinant antibody fragments bind human pancreatic islet cells

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ABSTRACT

We describe here the humanisation of two mouse monoclonal antibodies that bind to surface markers on human pancreatic islet endocrine cells. Monoclonal antibodies produced by the HIC1-2B4 and HIC0-4F9 mouse hybridomas bind distinct surface molecules expressed on endocrine cells and have been validated for a number of experimental methods including immunohistochemistry and live cell sorting by flow cytometry. Variable region framework and first constant region domain sequences were replaced with that from compatible human antibody sequences, and the resulting recombinant antigen-binding fragments were cloned and expressed in mouse myeloma cells. ELISA, fluorescent immunohistochemistry, and flow cytometry were used to assess the specificity of the humanised antibody fragments. Purification and binding analyses indicated that human islet endocrine cell binding was retained in the humanised antibody fragments. These humanised, recombinant antibody fragments have a lower risk of eliciting adverse responses from a patient's immune system and, therefore, have highly improved clinical potential. Thus, they may be used to image, target or carry cargo specifically to islet cells in human patients.

1. Introduction

Beta cells in the pancreatic islets play a critical role in regulating blood glucose metabolism. This regulation is lost as beta cells are targeted and destroyed by the immune system in Type 1 diabetes (van Belle et al., 2011). Deregulation also occurs in Type 2 diabetes when beta cells become dysfunctional or when insulin-responsiveness is lost (Lin and Sun, 2010). More recent evidence has found that phenotypic and functional heterogeneity exist within the beta cell population itself (Dorrell et al., 2016). Maintaining the balance between these subsets may have important implications for long term health. It is obvious that fully functional beta cells, comprising < 2% of the pancreatic mass, are vitally important to survival. Thus, the development of molecular tools capable of monitoring, and eventually treating, beta cells is of high priority.

Monoclonal antibodies (mAbs) have had a tremendous impact on biomedical research and biomedicine. Their unique ability to bind to ligands or protein epitopes with specificity and high affinity lends itself to countless applications, particularly as antibodies can be labeled for detection or coupled to other molecules for added functionality (Nieri et al., 2009; Perez et al., 2014). The specificity of hybridoma-produced monoclonal antibodies is stable over time and, since these hybridomas are immortalized cells, mAbs can be produced in continuous supply (Kohler and Milstein, 1975). Because the vast majority of mAbs are rodent-derived, however, clinical use has been limited due to serious consequences of immunoreactivity as well as rapid clearance upon re-administration (Hoffman, 1990; Tjandra et al., 1990). Humanisation of rodent mAbs may be accomplished by replacing the variable framework residues and constant regions with their human counterpart, thus leaving the antibody combining site (encoded by the complementarity-determining regions, CDRs) as the only region of rodent origin (Ahmadzadeh et al., 2014; Jones et al., 1986; Riechmann et al., 1988). Spurred by advances in technology, humanised as well as fully human mAbs (Bruggemann et al., 2015; Green et al., 1994; Lonberg et al., 1994; McCafferty et al., 1990) are now one of the fastest growing categories of therapeutic drugs in the clinical market today (Beck et al., 2010; Nelson et al., 2010). The role of mAbs in the clinical setting has been diverse, extending from diagnostic imaging of tumors to the delivery of conjugated toxins to specific tissues or cell types (Keenan et al., 1985; Luo et al., 2011; Teicher and Chari, 2011). Although the majority

Abbreviations: APC, allophycocyanin; CDR, complemetarity determining region; Cy3/Cy7, cyanin 3/cyanin 7; Fab, antigen-binding fragment of immunoglobulin; Fc, constant region fragment of immunoglobulin; Fd, heavy chain constant region of Fab; HC, heavy chain of immunoglobulin; HRP, horse radish peroxidase; IgG, immunoglobulin gamma; IgM, immunoglobulin mu; LC, light chain of immunoglobulin; mAb, monoclonal antibody; PDB, Protein Data Bank; PE, phycoerythrin; TMB, 3,3′,5,5′-tetramethylbenzidine; VH, variable region, heavy chain of immunoglobulin; VL, variable region, light chain of immunoglobulin

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of antibody-based drugs currently in use are approved for the treatment of cancer or immune disorders (Chan and Carter, 2010; Weiner et al., 2010), with the exponential growth in the number of therapeutic antibodies currently in clinical trials, many additional disorders may soon be targeted.

In previous studies, we generated a number of mouse mAbs that bind to human islet endocrine cell surface antigens (Dorrell et al., 2008). These mAbs have been validated in multiple experimental settings including flow cytometric staining and isolation of human islet cell subpopulations (Dorrell et al., 2016; Dorrell et al., 2008), immunohistochemical staining of islets in pancreatic tissue sections (Dorrell et al., 2008; Bramswig et al., 2013; van der Meulen et al., 2012), and *in vivo* labeling of human islets transplanted into mice (Buenafe et al., manuscript submitted). The HIC1-2B4 and HIC0-4F9 mAbs are of particular interest as they recognize surface antigens present on beta cells (Dorrell et al., 2008). Engineering these mouse mAbs to be compatible for use in human patients could generate tools capable of islet-specific imaging or directed delivery to human beta cells in patients. Thus, these reagents have therapeutic potential for mediating beta cell recovery or protection in Type 1 and Type 2 diabetes. In the current paper we describe the humanisation of the HIC1-2B4 and HIC0-4F9 mAbs, and their generation as recombinant antigen-binding fragment (Fab) antibody molecules.

2. Materials and methods

2.1. Antibodies and reagents

HIC1-2B4, HIC0-4F9 and HIC3-2D12 mAbs were generated as described (Dorrell et al., 2008). Mouse mAbs to human kappa light chain (HP6053) and human IgG heavy chain (Fd region, HP6045) were obtained from Invitrogen|Thermo Fisher Scientific (Carlsbad, CA, USA); human proinsulin-specific mAb GN-ID4 from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA; dsbh.biology.uiowa.edu); human/mouse glucagon-specific mAb MAB1249 from R&D Systems, Inc. (Minneapolis, MN, USA); goat anti-human IgG H + L, goat anti-human IgG H + L-HRP, goat anti-mouse Ig LC-HRP, goat anti-mouse Ig LC-Cy3, goat anti-mouse IgG-Alexa 488, goat anti-mouse IgG-APC, goat anti-mouse IgM-Cy3, and goat anti-mouse IgM-PE from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA); goat anti-rat IgG-PE-Cy7 and goat anti-mouse IgG2a-DyLight 488 from BioLegend (San Diego, CA). Recombinant human CD98 was obtained from ACRO Biosystems (Newark, DE, USA); recombinant human CD200 from Life Technologies|Thermo Fisher Scientific (Carlsbad, CA, USA). Enzymatic digestion of mAbs was performed with the Pierce Mouse Fab Preparation Kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Flow cytometry

Human pancreatic islets (obtained through the Integrated Islet Distribution Program, City of Hope, Duarte, CA, USA; https://iidp.coh.org/) were washed with PBS and dispersed into a single cell suspension after incubation with Hyclone trypsin (GE Healthcare, Chicage, IL, USA). For surface staining, islet cells were incubated with islet-specific primary mAbs for 1 h, followed by incubation with labeled secondary antibodies for 30 min in RPMI +5% FBS. For HIC1-2B4 and HIC0-4F9 mAbs, goat anti-mouse IgG-APC antibody was used as the secondary antibody. For HIC3-2D12 mAb, goat anti-mouse IgM-PE secondary antibody was used. For subsequent intracellular staining with hormone-specific mAbs, surface-stained cells were fixed with 2% paraformaldehyde, permeabilized, and then blocked with 2% FBS. Cells were stained with primary mAb (anti-proinsulin, anti-glucagon or isotype control mAb) in 1× Perm buffer (eBioscience Inc., San Diego, CA, USA) for 1 h followed by goat anti-rat IgG-PE-Cy7 or goat anti-mouse IgG2a-DyLight 488 secondary antibody. Samples were analyzed on an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA). Non-viable cells were excluded with the use of the LIVE/DEAD aqua fixable stain (Life Technologies). Gating strategy using FlowJo analytical software version 10.1r7 (FlowJo LLC, Ashland, OR, USA): After exclusion of non-viable cells and debris, samples were gated to exclude doublets and larger aggregates. For back-gating of intracellular staining, proinsulin or glucagon positive cells were gated separately and then superimposed upon contour plots displaying surface stained islet cell populations.

2.3. ELISA for antigen binding

ELISA wells were coated with 300 µM antigen (CD98, CD200, or BSA) overnight in bicarbonate coating buffer, pH 9.6, blocked with RPMI +5% FBS, then serially diluted IgG antibody or recombinant Fab (rFab) was added and incubated for 1 h. Binding of mouse IgG mAb was detected with goat anti-mouse Ig LC-HRP followed by TMB substrate (BD Biosciences, San Diego, CA, USA). For CD98-coated wells, rFab binding was detected with goat anti-human IgG H + L-HRP followed by TMB substrate. CD200-coated wells had high background reactivity with the goat anti-human IgG H + L-HRP. Therefore, the anti-human Fd mAb was used as secondary antibody, followed by goat anti-mouse Ig LC-HRP and TMB for detection. For increased binding avidity, rFabs were dimerized by pre-incubating for 1 h with the anti-human Fd mAb at a molar ratio of 1:0.4 (rFab:mAb) before being added to antigen-coated wells.

2.4. ELISA for screening of rFab transfectants

ELISA wells were coated overnight with anti-human IgG H + L, anti-human Fd mAb (each at 10 µg/ml) or anti-human kappa LC (at 25 µg/ml). After blocking with RPMI +5% FBS, supernatants from growth-positive transfectants were incubated for > 1 h, followed by detection with goat anti-Hu IgG H + L-HRP and TMB substrate.

2.5. Fluorescent immunohistochemistry (IHC-Fl)

Staining of human pancreatic tissue sections was performed by incubating with 100 µl of mAb or rFab in RPMI +5% FBS as primary antibody. Slides were washed in PBS and secondary antibody was applied at a 1:200 dilution. For HIC1-2B4 and HIC0-4F9 mAbs, goat anti-mouse IgG-Alexa 488 antibody was used as the secondary antibody. For HIC3-2D12 mAb, goat anti-mouse IgM-Cy3 secondary was used. For rFab, incubation with anti-Hu Fd mAb was followed by detection with goat anti-mouse Ig LC-Cy3. After washing, all sections were mounted in a solution containing 10% glycerol and 4% N-propyl gallate (Sigma–Aldrich, St. Louis, MO, USA) with 0.001% Hoechst 33,342 as a nuclear label prior to evaluation with a Zeiss LSM 700 confocal microscope with Zen image analysis software (CarlZeiss, Jenna, Germany).

2.6. Molecular cloning of Ig V genes

RNA was isolated from hybridoma cells expressing the mAbs of interest using the RNeasy Mini Prep kit (Qiagen, Germantown, MD, USA) and cDNA was synthesized using oligo(dT) and SuperScript II RT (Invitrogen). V-region sequences were PCR-amplified using the mouse VH, VL and constant-region primer sets published by Kettleborough et al. (Kettleborough et al., 1993). Amplification reactions were performed as described, with modifications. Annealing temperatures were found to be optimal at 60° for VH and 62° for Vκ in reactions using Cloned Pfu Polymerase (Agilent Technologies, Santa Clara, CA, USA), with cycle number varying from 25 to 28. PCR products (650–700 bp) were gel-purified in 1% agarose and purified for sequencing using the Qiaex II Gel Extraction kit (Qiagen). Sequencing was completed at the OHSU DNA Services Core using the following primers (Cγ: 5′-CCYTTGACMAGGCATCCYAGDGTC; Cκ:5′-GTTGTTCAAGAAGCACACGACTGAGGC). All PCR products generated clean sequences of at least 400