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Research paper

Development of an enzymatic assay for the detection of neutralizing antibodies against therapeutic angiotensin-converting enzyme 2 (ACE2)

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ABSTRACT

Therapeutic proteins have the potential to elicit immune responses in animals and humans (Mire-Sluis et al., 2004; Yu et al., 2006; Shankar et al., 2008). Contributors to the response could include product related factors such as chemical modifications, impurities that co-purify with product, contaminants, formulation, aggregates, and clinical factors such as dose concentration, dosing frequency, route of drug administration, rate of administration, patient underlying disease, concomitant medication, and genetic status among others (Patten and Schellekens, 2003). Further, an immune response triggered by a therapeutic enzyme may neutralize the endogenous counterpart resulting in a decrease or depletion of the therapeutic and endogenous enzymes imposing safety concerns for patients. Therefore, monitoring of anti-drug antibody (ADA) and neutralizing antibody (NAb) responses to both the recombinant therapeutic enzyme and endogenous enzyme is important during early development and subsequent clinical studies. Testing considerations for NAb detection against therapeutic enzymes have been published mostly for lysosomal storage diseases (Wang et al., 2008). NAb cross-reactivity to the endogenous counterpart has also been characterized (Sominanda et al., 2010). Here, we describe an enzymatic NAb assay which detects neutralizing antibodies to both recombinant and endogenous angiotensin-converting enzyme 2 (ACE2). NAb assay sensitivity was optimized by selecting the assay incubation time as 20 min with an enzyme concentration of 0.5 μg/mL. Four anti-ACE2 antibodies out of a commercial panel of 18 were found to have neutralizing capabilities based upon their ability to abrogate ACE2 enzymatic activity. We demonstrated assay specificity by small peptide inhibitors specific for ACE or ACE2. DX600, an ACE2 specific inhibitor did not cross-react with ACE. Conversely, captopril, an inhibitor of ACE did not inhibit ACE2. The assay specificity for ACE2 neutralizing antibodies was further demonstrated by the lack of reactivity of two species control antibodies and 14 anti-ACE2 antibodies. Moreover, we demonstrated assay specificity to human endogenous ACE2 from human epithelial cells. Three human cell lines (Calu-3, Caco-2, Huh-7) were evaluated for the cell surface expression of ACE2 by flow cytometry and Western blot. Subsequently, whole cell lysates, cell culture supernatant, and live cells were evaluated in the assay. Results demonstrated that Calu-3 had elevated levels of ACE2 compared to Caco-2 or Huh-7. Calu-3 also demonstrated elevated ACE2 enzymatic activity in all three sources and could be inhibited by the ACE2 specific inhibitor DX600 as well as the neutralizing antibodies for the recombinant ACE2. Thus, we describe here a method to detect NAb against a therapeutic enzyme and assess NAb cross-reactivity to the native endogenous enzyme. The approach of method development described here could be applied for the assessment of NAb responses to other enzymatic therapeutics.

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1. Introduction

ACE and ACE2 are part of the renin-angiotensin system (RAS) which controls blood pressure, electrolytes, and intravascular fluid volume. Increased ACE activity has been associated with acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (Cooke et al., 2002). ACE2, a homolog to the carboxypeptidase ACE, functions as a counter-regulatory mechanism and thus may be a potential therapeutic for ALI/ARDS patients. ACE2 counterbalances the multiple functions of ACE by cleaving a single amino acid from angiotensin II (Ang II), forming Ang 1-7. Ang 1-7 has been associated with anti-inflammation and vasodilation. In the ARDS rat model, ACE activity was enhanced along with reduced levels of Ang 1-7. Therapeutic intervention with Ang 1-7 attenuated the inflammatory mediator response, markedly decreased lung injury scores, and improved lung function (Wosten-van Asperen et al., 2011). Thus, ACE2 offers a promising novel treatment modality for patients. However, the therapeutic enzyme may elicit an immune response of anti-drug antibodies (ADA) within dosed subjects (Mire-Sluis et al., 2004; Yu et al., 2006; Shankar et al., 2008). The presence of ADA could have multiple outcomes which could include: no impact, hypersensitivity/anaphylaxis, reduced bioavailability, or reduced clinical efficacy (Patten and Schellekens, 2003). In addition, an ADA response could cross-react and neutralize the native endogenous counterpart leading to the generation of autoimmunity. Thus, vigorous immunogenicity evaluation plans are warranted for such a therapeutic enzyme like ACE2, including the assessment of ADA/NAb cross-reactivity to native and the recombinant enzyme (Wang et al., 2008; Sominanda et al., 2010).

Cell-based or ligand binding assays are currently accepted methods for detecting neutralizing antibodies (NAb) directed against therapeutic antibodies and proteins. We previously published a cell-based method for NAb directed against a monoclonal antibody therapeutic (Liao et al., 2012). Here, we describe a method to determine NAb presence directed against the therapeutic enzyme rhACE2 and cross-reactivity with the endogenous counterpart enzyme. An enzymatic activity assay was chosen since this format directly assesses the catalytic activity of ACE2 and neutralization capacity of anti-ACE2 antibodies. We optimized NAb sensitivity by evaluating assay incubation time and enzyme concentration. We characterized enzyme neutralization with commercially available antibodies and a hyperimmunized monkey serum. We determined the assay to be ACE2 specific by evaluating enzyme specific inhibitor and also by determining that the homolog ACE was not reactive under these conditions. We further selected a human cell line with ample ACE2 expression and demonstrated that the positive control anti-ACE2 monkey serum was cross-reactive to human native endogenous ACE2. The assay was validated according to FDA guidance (FDA, 2009) and deemed suitable for clinical sample testing.

2. Materials and methods

2.1. Cell lines, reagents, ACE2 antibodies

Calu-3 (a human lung epithelial cell line), Caco-2 (human epithelial colorectal adenocarcinoma cell line), and Huh-7 (human hepatic cell line), were purchased from ATCC. Heat-inactivated fetal bovine serum (FBS) was purchased from SAFC Biosciences (Lenexa, KS). Dulbecco's Modified Eagle Medium (high glucose) (DMEM), Eagle's minimal essential medium (EMEM), PBS and Trypsin-EDTA were obtained from Invitrogen Corp. (Carlsbad, CA). Recombinant ACE2 was provided by GlaxoSmithKline BioPharm unit. ACE2 substrate Mca-APK-Dnp and ACE2 specific inhibitor (DX600) were purchased from AnaSpec (Fremont, CA). Recombinant ACE was purchased from R&D systems (Minneapolis, MN). ACE inhibitor, captopril was purchased from Sigma (St. Louis, MO). Normal human sera were purchased from Bioreclamation (Hicksville, NY). The ACE2 antibodies and the sources were listed in Table 1.

2.2. Measurement of cellular ACE2 by Western blotting and flow cytometry

Confluent cells were trypsonized and collected by centrifuging at 2000 rpm for 10 min. Cell pellets were washed once with cold phosphate-buffered saline and lysed with Mammalian Protein Extraction Reagent (M-PER, Thermo Scientific) containing protease inhibitor (Roche) on ice for 20 min. Lysates were centrifuged at 12,000 rpm for 10 min. The supernatant was collected and protein concentrations were determined by BCA kit (Pierce) using bovine serum albumin as standard. 50 μg of cell lysate, 32.5 μL cell culture supernatant, and 1 ng rhACE2 protein were resolved by NuPAGE 4–12% gradient Bis-Tris gels in MOPs buffer then transferred to a nitrocellulose membrane in 1× transfer buffer (Invitrogen) with 10% (v/v) methanol. The membrane was saturated with Odyssey blocking buffer at room temperature for 30 min then incubated with anti-ACE2 antibody (AF933, R&D systems) at room temperature for 1 h. IRDye680 (red, Li-Cor Bioscience) conjugated anti-goat secondary was used at 1:7500 dilution and incubation was 30 min at room temperature. The membrane was reblotted with anti-GAPDH (mAb374, Millipore) detected with IRDye800 (green, Li-Cor Bioscience) anti-mouse secondary antibody at 1:7500. Bound antibodies were detected by Odyssey.

Cell surface ACE2 was detected by flow cytometry. Briefly, cells were fixed by 4% paraformaldelhyde on ice for 20 min then washed twice in assay buffer (1× PBS with 1% BSA and 2 mM EDTA). Cells were resuspended in assay buffer containing 1 μg/mL anti-ACE2 antibody (AF933, R&D systems) and incubated on ice for 30 min. Cells were washed twice with assay buffer, resuspended in assay buffer containing 0.5 μg/mL PE conjugated donkey anti-goat (Abcam, Cambridge, MA) and incubated on ice for 20 min. Cells were washed and resuspended for flow cytometry analysis.

2.3. ACE2 enzymatic activity kinetics

The catalytic activity of recombinant ACE2 and cellular ACE2 was measured by hydrolysis of a highly specific quenched fluorescent substrate, 7-methoxycomarin-4-yl acetyl-Ala-Pro-Lys-(2,4-dinitrophenyl)-OH (Mca-APK-Dnp) (AnaSpec, Fremont, CA). rhACE2 was serial diluted with dilution buffer (100 mM Glysine, 50 μM ZnCl, 150 mM, 1% BSA) starting with 5 μg/mL. The serial diluted enzyme was further diluted 1:5 with activity buffer (150 mM NaCl, 75 mM Tris, 10 μM $ZnCl_2$, 0.01% Triton X-100, pH 7.2). 50 μL of the diluted enzyme was transferred to a black 96-well plate, then 50 μL of substrate at 200 μM was added to each well (final

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