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A novel high-sensitivity electrochemiluminescence (ECL) sandwich immunoassay for the specific quantitative measurement of plasma glucagon

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ABSTRACT

Objectives: To develop a novel, dual-monoclonal sandwich immunoassay with superior sensitivity that provides a rapid and convenient method for measuring glucagon. Glucagon is a 29-amino acid polypeptide hormone produced in the pancreas by the α-cells of the islets of Langerhans. Working in concert with insulin, glucagon is involved in regulating circulating glucose concentrations.

Design and methods: The immunoassay utilizes Meso Scale Discovery (MSD) electrochemiluminescence (ECL) technology and two affinity-optimized monoclonal antibodies. A series of experiments was performed to determine the linear range of the assay and to evaluate sensitivity, accuracy, recovery, precision, and linearity.

Results: The sandwich assay was specific for glucagon and did not recognize the closely related peptide oxyntomodulin or other incretin peptides. The assay demonstrated excellent recovery, precision, and linearity, and a broad dynamic range of 0.14 pmol/L to 1950 pmol/L. In addition, assay results were highly correlated with those obtained using a previously described competitive RIA employing polyclonal antiserum.

Conclusion: The use of affinity-optimized monoclonal antibodies in a sandwich immunoassay format provides a robust, sensitive, and convenient method for measuring concentrations of glucagon that is highly sensitive and specific. This immunoassay should help to improve our understanding of the role of glucagon in the regulation of glucose metabolism.

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Introduction

Glucagon is a 29-residue polypeptide hormone that is produced in the pancreas by the α-cells of the islets of Langerhans [1]. The amino acid sequence of the peptide is completely conserved in mammals. Glucagon and glucagon-like peptides are transcribed from a common proglucagon gene that is expressed in the pancreas, intestine, and brain [2]. Glucagon is involved in maintaining normal levels of glucose in the blood by stimulating the liver to convert glycogen to glucose. This conversion of glycogen to glucose in concert with the opposing action of insulin to promote storage of glucose as glycogen enables the two hormones to tightly regulate blood glucose levels. Basal amounts of glucagon are essential for the maintenance of normoglycemia, and thus a key physiological role of glucagon is to prevent hypoglycemia. It has been proposed that improper expression of glucagon may contribute to the development of the hyperglycemia in diabetes [3].

Glucagon levels increase rapidly in response to hypoglycemia. Specifically, the glucagon response is the primary essential defense mechanism utilized by the body to restore blood glucose to normal levels. A complex interplay of signals is required for the proper regulation of glucagon secretion. These controlling factors which include glucose, intra-islet paracrine factors such as insulin and GLP-1, and the central and autonomic nervous systems interact in a coordinated fashion to regulate glucagon secretion [4,5]. In healthy subjects, glucagon levels increase in response to a high-protein meal and decrease in response to a high carbohydrate meal or oral glucose [6]. The glucagon receptor is predominantly expressed in the liver where its activation leads to increased glucose production. Glucagon receptors are also expressed at lower levels in many other tissues. Recent studies have also implicated glucagon as an important component in the process of regulation of energy metabolism [7].

A radioimmunoassay to measure glucagon was first described 50 years ago [8]. Since that time there have been many advances in immunoassay methods; however, these have not translated to a method that specifically measures glucagon without interference from cross-reacting materials [9]. In this study, we describe a highly specific electrochemiluminescent (ECL) sandwich immunoassay with superior sensitivity and recovery that provides a rapid and convenient method

Abbreviations: ECL, electrochemiluminescence; RIA, radioimmunoassay; GLP, glucagon like peptide; TBST, Tris buffered saline with 0.05% Tween-20; BSA, bovine serum albumin; PBS, phosphate buffered saline; WT, wild-type; MRD, minimum required dilution; scFv, single chain variable fragment; LLOQ, lower limit of quantitation; CDR, complementarity-determining region; SA-PE, streptavidin-R-phycoerythrin; SOE-PCR, single overlap extension PCR; VH: Ig, heavy chain variable region; VL: Ig, light chain variable region; MSD, Meso Scale Discovery; GaM-488, Alexa fluor 488 goat anti mouse IgG.

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for quantifying glucagon. Importantly, the assay utilizes two monoclonal antibodies for the specific measurement of plasma glucagon, allowing for sustainable production of the antibodies and therefore does not have to rely upon polyclonal antisera.

Methods

Human plasma samples

Blood samples from 6 healthy volunteers who gave written informed consent were collected into P800, EDTA or Serum collection tubes (Becton Dickinson, Franklin Lakes, NJ USA). After separating plasma or serum from cells, samples were stored at −70 °C prior to analysis of glucagon levels. Plasma (P800) samples for the determination of the normal range were collected from 58 healthy volunteers who gave informed consent. Plasma samples with a wide range of glucagon levels to compare RIA values with ECL assay values came from patients in a study who gave permission for their samples to be analyzed.

Affinity maturation of monoclonal antibodies

Both mid-domain and C-terminal anti-glucagon antibodies were optimized using *in vitro* directed evolution. The WT scFv genes, in which the antigen-specific VH and VL domains are linked into a single polypeptide were created by single overlap extension PCR (SOE-PCR). Spiked degenerate oligonucleotides were used for saturation mutagenesis of the VH and VL CDR [10,11]. An overrepresentation (≥10×) of each CDR library or selection output from a previous round of selection was incubated with biotinylated glucagon (Anaspec, Freemont, CA, USA) in selection buffer (PBS pH 7.4, 0.5% BSA) at 25 °C for 30 min prior to transfer to ice and washing with cold selection buffer. Bound antigen was detected using streptavidin-R-phycoerythrin (SA-PE) (1:200 dilution). Clones of interest were enriched using fluorescence-assisted cell sorting on a FACSAria cell sorter (BD Biosciences, San Jose, CA, USA). Individual clones from CDR libraries with improved binding from the third round of selection were sequenced.

A combinatorial library combining the CDR regions of clones from the individual libraries with improved binding was constructed by SOE-PCR, and subjected to further rounds of selection with increased stringency. Individual combinatorial clones were sequenced, and the relative binding affinity determined after antigen titration and/or dissociation assay. The resulting VH and VL genes were ligated into separate murine IgG or IgK expression vectors, respectively, to create the final optimized variants. IgG was purified by protein A after large scale transient transfection using HEK 293 cells.

Biacore analysis

Kinetic analysis and binding specificity of anti-glucagon IgG were determined using a Biacore T100 instrument (GE Healthcare Biosciences, Pittsburgh, PA, USA). A Series S Sensor Chip CM5 with 6000 response units of goat anti-mouse IgG capture antibody (Southern Biotech, Birmingham, AL, USA) covalently attached was used to capture 1500 response units of IgG diluted to 10 μg/mL in running buffer (HEPES buffered saline containing 3 mM EDTA and 0.05% Tween). Increasing concentrations of the glucagon were injected for 5 min at a flow of 30 μL/min followed by at least 10 min dissociation phase. The surfaces were regenerated following each dissociation phase with two 15 μL injections of glycine-HCl, pH 1.5 at a flow rate of 100 μL/min. Kinetic constants were determined using the T100 Evaluation software. Specificity was tested by injections of 100 nM oxyntomodulin, (Anaspec, Freemont, CA, USA) under the described assay conditions.

Antibody labeling

Monoclonal antibodies were adjusted to an approximate concentration of 2 mg/mL and were Biotin and MSD-SulfoTag (Meso Scale Discovery, (MSD) Gaithersburg, MD USA) labeled according to manufacturer's protocols. Capture antibody was biotin labeled with Thermo no-weigh EZ Link Sulfo-NHS-LC Biotin with a 20-fold molar excess of biotin. Conjugate antibody was labeled with MSD Sulfotag NHS Ester with a 12-fold molar excess of ruthenium. Following the labeling reactions, antibodies were extensively dialyzed to remove unbound label.

Glucagon ECL immunoassay

The glucagon sandwich assay was performed on MSD Streptavidin 96-well plates that were washed with three times with TBST (Tris buffered saline containing 10 mmol/L Tris pH 7.40, 150 mmol/L NaCl with 1 mL Tween 20/L) and blocked with 1% Ovalbumin (Sigma, St. Louis, MO, USA) in TBS for 1 hour at room temperature. Following washing of the plate, 50 μL of biotin-labeled capture antibody (1 μg/mL) was added and allowed to bind to the plate for one hour with gentle shaking. Afterward, the wells were washed three times with TBST, and 100 μL of glucagon standards consisting of varying concentrations of glucagon protein in assay buffer consisting of 50 mmol/L HEPES, pH 7.40, 150 mmol/L NaCl, 10 mL/L Triton X-100, 5 mmol/L EDTA, and 5 mmol/L EGTA and 1% ovalbumin, which was supplemented with 100 μg/mL Heterophilic Blocking Reagent (Scantibodies, Santee, CA, USA) were added to the wells to generate a calibration curve. Plasma samples were diluted 1:8 in the same assay buffer, added to their respective wells, and incubated for 2.5 hours at room temperature with gentle rocking. Following aspiration, wells were washed 3 times with TBST, and 50 μL of 1 μg/mL ruthenium-labeled conjugate glucagon-specific detection antibody was added to the wells, which were incubated for 2.5 hours at room temperature. The plate was again washed three times with TBST, and 150 μL of 2×-MSD Read Buffer T was added to the wells. The plate was then read on an MSD SECTOR Imager 6000 reader, which recorded ruthenium electrochemiluminescence. Concentrations of glucagon in samples were interpolated against a standard curve made up of reference standard glucagon (Eli Lilly and Company, Indianapolis, IN, USA) using a 4 PL fit (Meso Scale Discovery Workbench).

Data analysis

All data are expressed as means ± SEM. Meso Scale Discovery Workbench software was used for fitting assay calibration curves and interpolation of unknown values. Data were plotted with SigmaPlot version 11.0. Microsoft Office Excel 2007 was used for data analysis. A P value of ≤0.05 was considered to indicate statistical significance.

Results

Our aim in this study was to develop a sandwich immunoassay that specifically measured glucagon and did not recognize oxyntomodulin or other potential cross-reacting species. A panel of anti-glucagon antibodies was screened to identify a pair of reagents that could be used to develop a dual monoclonal-based assay (data not shown). Kinetic analysis of the selected antibodies indicated that a more sensitive assay could be developed using antibody engineering to improve the binding characteristics of the parental clones (Table 1). Mutations were systematically introduced into the individual complementarity determining regions (CDRs) of each antibody, and the resulting libraries were subjected to multiple rounds of selection with decreasing concentrations of antigen and/or increasing periods of dissociation to isolate clones with improved affinities.

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