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Generation of monoclonal antibodies and development of an immunofluorometric assay for the detection of CUZD1 in tissues and biological fluids

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ABSTRACT

Background: CUB and zona pellucida-like domain-containing protein 1 (CUZD1) was identified as a pancreas-specific protein and was proposed as a candidate biomarker for pancreatic related disorders. CUZD1 protein levels in tissues and biological fluids have not been extensively examined. The purpose of the present study was to generate specific antibodies targeting CUZD1 to assess CUZD1 expression within tissues and biological fluids.
Methods: Mouse monoclonal antibodies against CUZD1 were generated and used to perform immunohistochemical analyses and to develop a sensitive and specific enzyme-linked immunosorbent assay (ELISA). CUZD1 protein expression was assessed in various human tissue extracts and biological fluids and in gel filtration chromatography-derived fractions of pancreatic tissue extract, pancreatic juice and recombinant protein.
Results: Immunohistochemical staining of CUZD1 in pancreatic tissue showed that the protein is localized to the acinar cells and the lumen of the acini. Western blot analysis detected the protein in pancreatic tissue extract and pancreatic juice. The newly developed ELISA measured CUZD1 in high levels in pancreas and in much lower but detectable levels in several other tissues. In the biological fluids tested, CUZD1 expression was detected exclusively in pancreatic juice. The analysis of gel filtration chromatography-derived fractions of pancreatic tissue extract, pancreatic juice and recombinant CUZD1 suggested that the protein exists in high molecular weight protein complexes.
Conclusion: This study describes the development of tools targeting CUZD1 protein, its tissue expression pattern and levels in several biological fluids. These new tools will facilitate future investigations aiming to delineate the role of CUZD1 in physiology and pathobiology.

1. Introduction

CUB and zona pellucida-like domain-containing protein 1 (CUZD1) was identified as a highly pancreas-specific protein and was proposed as a candidate biomarker for pancreatic related disorders [1,2]. The information on CUZD1 protein is sparse: The CUZD1 gene, also known as the uterine-ovarian specific gene 44 (UO-44) [3] and the estrogen–regulated gene 1 (ERG1) [4], is mapped/resides at/on chromosome 10q26.13 and encodes for a 608-amino acid polypeptide containing a signal peptide, two complement subcomponents C1s and C1r, Uegf, Bmp1 (CUB) domains, one zona pellucida (ZP) domain, a single-spanning transmembrane region and a short cytoplasmic tail [5]. The

Abbreviations: BSA, bovine serum albumin; CUB, complement subcomponents C1s and C1r, Uegf, Bmp1; CUZD1, CUB and zona pellucida-like domain-containing protein 1; ELISA, enzyme-linked immunosorbent assay; ERG1, estrogen–regulated gene 1; GP2, glycoprotein 2; HRP, horseradish peroxidase; (LC-MS/MS), liquid chromatography-tandem mass spectrometry; (PVDF), polyvinylidene difluoride; UO-44, uterine-ovarian specific gene 44; ZP, zona pellucida

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protein is predicted to be heavily glycosylated and is highly conserved among species [6]. CUZD1 mRNA undergoes a complex series of alternative splicing events that give rise to three protein isoforms. The largest isoform, approximately 607 amino acids long (68 kDa), contains the entire gene while the other two isoforms, 326 amino acids (37 kDa) and 241 amino acids (28 kDa) long, contain only the ZP domain and the transmembrane region or half of the ZP domain and the transmembrane region, respectively [5]. CUB and ZP domains occupy the majority of the extracellular region of the largest isoform and are known to be involved ligand binding, oligomerization and cell adhesion [7,8].

While the exact biological function of CUZD1 function is still unknown, it has been recently linked to Inflammatory Bowel Disease as it is one of the two main targets of pancreatic autoantibodies which are novel serological markers for Crohn's disease. According to previously published studies generated by using indirect immunofluorescence, pancreatic autoantibodies against CUZD1 are detected in 22–26% of patients with Crohn's disease and in 11–15% of patients with ulcerative colitis [9–11]. Using a novel in house developed enzyme-linked immunosorbent assay (ELISA) targeting CUZD1 autoantibodies, Farkona et al. was able to detect them in 16% of patients with Crohn's disease and in 9% of Ulcerative Colitis patients [12]. Previous studies implicate the protein as having a role in cancer progression [5,13]. In 2001, Huynh et al. isolated a uterine and ovarian specific, tamoxifen and estrogen-induced rat UO-44 cDNA (the ortholog of human CUZD1) through differential display and cDNA library screening [3]. The authors found that the UO-44 mRNA transcript was observed only in the uterus and ovary of rats. In 2004, the same group reported the cloning and characterization of four novel splice variants of the human ortholog of UO-44 (CUZD1) [2]. While the authors found in 2001 that the Rat UO-44 was highly expressed in the ovaries and uterus [3], the human ortholog CUZD1 was found highly expressed in the pancreas [5]. In the same study, they also reported the overexpression of CUZD1 in the majority of ovarian tumors. In a more recent study, Mapes et al. have shown that in mice CUZD1 is also expressed in mammary ductal and alveolar epithelium and that it has a pivotal role in JAK/STAT5 signaling that regulates mammary gland development during pregnancy [14].

Thus far, CUZD1 protein levels in tissues and biological fluids have not been extensively examined due to the lack of suitable reagents and techniques. Therefore, the purpose of the present study was to develop recombinant protein, specific antibodies, and an ELISA immunoassay to assess CUZD1 expression at the tissue level and its concentration in various biological fluids.

2. Materials and methods

2.1. Monoclonal antibody production

Monoclonal antibodies were produced as previously described [15]. Briefly, female BALB/c mice were purchased from Jackson laboratories via the Toronto Centre for Phenogenomics (TCP). All animal research was approved by the TCP Animal Care Committee. Mice were injected subcutaneously with 100 μg of human recombinant CUZD1 protein, mixed (1:1) with Sigma Adjuvant System (Sigma-Aldrich). Two subsequent booster injections with 20 μg of antigen in adjuvant were performed at three-week intervals. The final boost was an intraperitoneal injection with 20 μg of antigen in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4). Three days later, the mouse spleen was excised aseptically and homogenized. Extracted spleen cells were fused with NSO murine myeloma cells (5:1 ratio) using polyethylene glycol (Sigma-Aldrich). Successfully fused cells were isolated using selection medium, containing 2% Gibco® HAT supplement (Thermo Fisher Scientific, Waltham, MA) and 20% fetal bovine serum (Hyclone, GE Healthcare).

2.2. Screening for immunogen-reacting clones by an IgG capture ELISA

Seven hundred and fifty hybridoma clones were screened for reactivity against the recombinant protein by using an IgG capture ELISA, as previously described [16].

2.3. Expansion of hybridomas and purification of monoclonal antibodies

Following the screening procedure, 21 hybridoma clones shown to strongly react with the recombinant protein were further grown and transferred in serum-free media (Thermo Fisher Scientific), containing 8 mM L-Glutamine. Supernatants were collected and purified using a protein G column (GenScript, Piscataway NJ, USA). Briefly, culture supernatants were diluted two times in binding buffer (20 mM NaH_2PO_4, 150 mM NaCl, pH 8.0) and loaded onto the column. The column was then washed with the binding buffer and antibodies were eluted with 0.1 M glycine at pH 3.0. The purified antibodies were then screened against both the recombinant protein and pancreatic tissue extracts by the ELISA described in the next section. Clones 275 and 119 were chosen to be used as the coating and the detection antibody in our ELISA, respectively.

2.4. ELISA development

White polystyrene microtiter plates were incubated overnight at room temperature with 100 μL of coating antibody solution containing 500 ng of monoclonal anti-CUZD1 antibody (clone 275) diluted in 50 mmol/L Tris buffer (pH 7.8). The plates were then washed three times with the washing buffer (20 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.4). CUZD1 calibrators or samples, diluted in a bovine serum albumin (BSA) solution [60 g/L BSA, 50 mmol/L Tris (pH 7.80) and 0.5 g/L sodium azide], were then pipetted to each well (50 μL/well) along with 50 μL of assay buffer A (60 g/L BSA, 25 mL/L normal mouse serum, 100 mL/L normal goat serum, and 10 g/L bovine IgG in 50 mM Tris, pH 7.8, 0.005% (v/v) Tween-20) and incubated for 2 h with shaking at room temperature. The plates were washed three times with the washing buffer after which 100 μL of biotinylated detection antibody solution containing 200 μg/L anti-CUZD1 monoclonal antibody 119 in assay buffer A was added to each well and incubated for 1 h at room temperature with shaking. The plates were washed six times with the wash buffer. Subsequently, 100 μL of alkaline phosphatase-conjugated streptavidin (50 ng/mL in BSA solution) were added to each well and incubated for 15 min with shaking at room temperature. Plates were washed as above and 100 μL of diflunisal phosphate solution [0.1 mol/L Tris-HCl buffer (pH 9.1) containing 1 mmol/L diflunisal phosphate, 0.1 mol/L NaCl and 1 mmol/L MgCl2] was added to each well and incubated for 10 min with shaking at room temperature. Developing solution (100 μL, containing 1 mol/L Tris base, 0.4 mol/L NaOH, 2 mmol/L TbCl3, and 3 mmol/L EDTA) was pipetted into each well and mixed for 1 min. Time-resolved fluorescene was measured with the Wallac EnVision 2103 Multilabel Reader (Perkin Elmer, Waltham, MA, USA).

Recombinant CUZD1 protein, produced and purified as described elsewhere [12], was used to generate the calibration curve. CUZD1 calibrators were prepared by diluting the purified recombinant CUZD1 in the general diluents. These calibrators were used to define the detection limit of the assay. To determine the linearity of the CUZD1 immunoassay, we serially diluted a pancreatic tissue extract in BSA solution and measured the CUZD1 concentration with the standard assay procedure. Recombinant CUZD1 was added to normal sera at different concentrations and measured with the developed CUZD1 immunoassay. Recoveries were calculated after subtraction of the endogenous concentrations.

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