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A targeted proteomic approach for the identification of tumor-associated membrane antigens using the ProteomeLab™ PF-2D in tandem with mass spectrometry

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Abstract

Mapping differential expression of soluble proteins has become fairly routine using chromatofocusing in combination with the reversed-phase HPLC (ProteomeLab™ PF-2D by Beckman Coulter Inc.); however, identification of membrane antigens has not been reported thus far. In this report, we demonstrate a targeted proteomic approach employing immunoprecipitation, prior to 2D-LC separation, in tandem with MS/MS that can be used to identify tumor-associated membrane antigens. This system is very sensitive and reproducible in that only 1/4th the amount of starting material is required for analysis as compared to gel-based analysis, and permits a focused environment for eliminating non-specific interactions leading to an accurate resolution of the cognate antigen. This system also circumvents the well-known limitations associated with gel-based approaches. This approach has been validated in the identification of ErB2/HER-2 and was subsequently used to identify CD44E as the cognate antigen for VB1-008, one of our fully human, tumor-specific, monoclonal antibodies.
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The isolation of tumor-specific antibodies produced by cancer patients has been our approach in the design of antibody-based immunotherapeutics, for the treatment of patients with solid tumors. Identification of cognate antigens for such antibodies would enable a treatment regimen to be further tailored and hence provide greater clinical benefit for the patient. The complexity of cell-surface membrane antigens renders routine isolation and purification strategies difficult [1,2]; and attempts to separate intact proteins have been limited to only a small group of soluble proteins [3,4]. Although 2D-PAGE is capable of resolving 1500–3000 proteins in a single separation step, the separation and visualization of higher molecular weight, hydrophobic membrane proteins is generally not possible [5–7].

The ProteomeLab™ PF-2D system works on a principle similar to two-dimensional gel analysis, but uses a (gel-free) liquid phase approach. Hence, the critical parameters necessary for the separation of proteins, i.e., determining isoelectric points, resolving isomers, and their separation on the basis of hydrophobicity, have made antigen fractionation on a PF-2D system more successful and informative when compared to the other approaches [8]. A combination of PF-2D separation followed by final detection by SDS–PAGE has also been suggested for membrane proteins [2,9], but the well-known deficiencies of gel-based approaches still limit these procedures. To date there are no reports of any effective method for the identification of tumor-associated membrane antigens.

Here, we present the applicability of PF-2D fractionation for the separation of membrane antigens and membrane-associated antigens; when coupled to mass

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spectrometry and an additional pre-fractionation step. The method is not only sensitive and reproducible (uses 1/4th the protein concentration required for a gel-based approach), but also is easier to detect, analyze, and compare reactive versus non-reactive antigens simultaneously. In this study, we have used our approach to identify the HER-2 antigen, using a commercial anti-HER2 antibody, as a proof-of-concept, followed by the isolation and identification of CD44E, the cognate antigen for VB1-008, a human IgG Mab, as an example of one of the many antibodies successfully identified by this method. The results suggest that such a system could be used routinely for the rapid and efficient identification of membrane antigens.

Materials and methods

Tumor cell lines and antibodies. SKBR-3 (breast adenocarcinoma) and Hep-G2 (hepatocellular carcinoma) were purchased from ATCC and cultured in accordance with the guidelines and recommendations supplied. Cells were grown and harvested at 90% confluence. SKBR-3 and Hep-G2 were the HER-2-positive and HER-2-negative cell lines, respectively, and anti-HER-2 was purchased from EMD Biosciences (Cat# OP15SP1). VB1-008, an IgG Mab, was generated from the PBLs of a breast cancer patient using our proprietary platform technologies. Anti-CD44 (parent) was purchased from R&D systems (Cat# BBA10); anti-CD44V3 (Cat # NCL-CD44V3); and anti-CD44-isoform2 (parent) (Cat# NCL-CD44 -2) were purchased from Vision Biosystems, anti-AFP (monoclonal) was purchased from Research Diagnostics (RDI—Cat# TRK4F16 –5H7) and recombinant h AFP was purchased from Calbiochem/EMD Biosciences, (Cat# 341498).

Membrane protein extraction. Approximately 500 μg of membrane protein, isolated from SKBR-3 and Hep-G2 cells using a combination of detergent-free lysis and differential centrifugation, was used for immunoaffinity purification. Briefly, cells were lysed in 250 mM sucrose, 1 mM EDTA, and 20 mM Hepes, pH 8.0, with a Wheaton homogenizer in the presence of protease inhibitors (Leupeptin, Sigma Cat# L9783; Pepstatin, Sigma Cat# P5318; Aprotinin, Bayer, Cat# 96-070-3; Bestatin, Sigma Cat# B8385; and PMSF, Sigma Cat# P7626 at 0.6 μg/mL concentration) and homogenate centrifuged at 200*g* to free the lysate of cell debris. The crude lysate was then centrifuged at 1000*g*, 4000*g*, and 200,000*g* to sediment nucleus, mitochondria, and membranes, respectively. The final membrane proteins were solubilized in a buffer containing 20 mM Hepes (pH 8.0) + 0.5% octyl-β-glucoside (OBG) + protease inhibitors.

Immunoprecipitation. A pre-clearing step using Protein-G Sepharose alone was the first step in the immunoprecipitation prior to the addition of the antibody. A total of 15–20 μg of antibody was used as the precipitating agent in the mixture. The antigen–antibody mixtures were nutated overnight at 4 °C using a buffer containing 10 mM Hepes, pH 8.0, 0.5% CHAPS, 1.2 mM EDTA, 150 mM NaCl, and 0.01% SDS in the presence of 0.6 μg/mL protease inhibitors. Immune complexes were centrifuged, washed with RIP-A lysis buffer, and eluted with 0.2 M glycine, pH 2.5. The eluate was neutralized with 1 M Tris base and prepared for the chromatofocusing run. All the chemicals used in the study were purchased from Sigma–Aldrich Company, 3050 Spruce Street, St. Louis, Missouri, 63103; and all electrophoresis supplies were from Invitrogen Canada Inc., 2270 Industrial St., Burlington, Ontario, L7P 1A1.

PF-2D fractionation of SKBR-3 and Hep-G2. Using the material generated from the above-mentioned pre-fractionation step, first dimension fractionation on a chromatofocusing HPCF column and the second dimension on a reversed-phase, HPRP column were performed, according to manufacturer's instructions and as reported previously [9]. The cognate binding antigens were eluted with glycine, neutralized, equilibrated with CF start buffer, and separated on the HPCF column using a pH gradient (8.5–4.8). All buffers and columns were purchased from Beckman Coulter Inc., Fullerton, CA. Fractions representing p$I = 5.1 \pm 0.2$ from HER-2-positive and -negative cell lines and p$I = 5.3 \pm 0.2$ from VB1-008-positive and -negative cell lines, respectively, were selected and fractionated on the HPRP column on the basis of their hydrophobicities.

Fractionation Analysis using ProteoVue™/DeltaVue™ software. Chromatographic profiles obtained for the HPRP column fractions were imported into ProteoVue™ for the final analysis using DeltaVue™. The analyses were combined for the antigen fractionation from both reactive and non-reactive cell lines to generate a comparative antigen expression map for each of the cell lines. Comparative profiling from both antibody-reactive and non-reactive cell lines were performed simultaneously, that clearly visualized differential expression more readily for better analysis.

Dot blot assay. Fractions from the HPRP columns from HER-2-positive and -negative cell lines were concentrated using microcon membranes and spotted on nitrocellulose membrane using a standard dot blot manifold (Schleicher and Schull) and probed with anti-HER-2 and signal detected by chemiluminescence. One blot for each cell line was processed along with SKBR-3 total membranes (5 μg) probed with anti-HER-2 (positive control) and mouse IgG (isotype-matched control). SDS–PAGE and Western blotting subsequently analyzed positives.

Western blot analysis. Fractions (HER2) from approximately eight identical runs of PF-2D were pooled, concentrated, and analyzed by SDS–PAGE and Western blotting. Pre-cast 10% Tris–glycine gels from Novex (Invitrogen) were used to separate proteins that were subsequently transferred onto nitrocellulose membranes for 40 min, electrophoretically. Membranes were blocked overnight in phosphate-buffered saline (PBS) containing 0.5% Tween (PBST) at 4 °C, washed extensively to remove blocking buffer, and incubated with anti-HER-2 or any other antibody at RT for 2 h. Blots were subsequently washed sequentially with PBST and PBS, followed by incubation with secondary antibody coupled to HRP for 30 min at RT. After thorough washing with PBST and PBS, blots were developed with ECL reagent (Amersham Biosciences) and visualized by Chemiluminescence. Images were captured on X-ray films (Kodak MR Film) and developed using a Kodak X-OMAT machine.

2D-PAGE and Western blot analysis. VB1-008 immunoprecipitates were concentrated up to 80 μL and buffered with equal volumes of rehydration buffer (Bio-Rad Cat# 163–2016). The protein solution was loaded onto IPG strips (4–7, Bio-Rad Cat# 163–2001) and subjected to isoelectric focusing on a Protean IEF (Bio-Rad Cat# 165–4000), according to manufacturer's instructions. Following the resolution of proteins on the basis of isoelectric points (p*I*), the IPG strip was reduced, alkylated (as per manufacturer's instructions), and separated on a second dimension SDS–PAGE gel. After 3 h of electrophoresis at 200 V, proteins from the gel were electro-blotted onto PVDF membranes overnight. The Western blots were then probed with anti-AFP and anti-CD44 (20 μg/mL), and reactivity detected by chemiluminescence.

Antigen identification. Fractions containing differentially expressed protein peaks in the positive cell line, SKBR-3, along with corresponding fractions from Hep-G2 that eluted from the HPRP (second dimension) column at 27, 28, and 29 min; and MDA-MB-435S, along with Panc-1 that eluted from HPRP at 16 and 17.8 min, respectively, were subjected to peptide extraction by in-solution tryptic digestion.

Peptide extraction using in-solution digestion. Fractions with identical retention times from both cell lines were processed simultaneously. HPRP fraction volumes were concentrated to a final volume of 100 μL, followed by standard reduction and alkylation using DTT (Sigma Cat# D-8225) and Iodoacetamide (Sigma Cat# I-1149). The samples were then subjected to tryptic digestion at 37 °C for 4–6 h. Peptide samples were then desalted, cleaned, and concentrated using μC18-zip tips (from Millipore Corporation, MA) and analyzed on API-QSTAR pulsar-i mass spectrometer (Hybrid-Quadrupole-TOF LC–MS/MS mass spectrometer, model—026026K, ABI/MDS Sciex, Concord, ON, Canada), equipped with a nanosource. The flow rate was 500 nL/min and spectra were obtained in LC-mode (information-dependent acquisition). The IS voltage was set at 1400 V, CID at 48 V, and pulsar frequency = 6.991 kHz. Spectra were calibrated with Glufibrinopeptide B (Sigma cat # F-3261) at ~5 pmol/μL.

Automated LC–MS/MS analyses were completed using a CapLC (Agilent HP1100) interfaced with a QqTOF-MS (ABI/MDS Sciex, Concord, ON, Canada), with a 150 mM by 300 μM i.d. column packed with

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