Journal of Circulating Biomarkers

OPEN ACCESS
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Identification of Immunoreactive Tumour Antigens Using Free and Exosome-Associated Humoral Responses

Original Research Article

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Received ; Accepted 21 November 2013

Abstract

Altered tumour antigens can initiate cellular and humoral immune responses; however, they often fail to eliminate tumours. In humans, the presence of cancer is generally associated with the suppression of T cell activation and effector responses, characterized as a Th1 to Th2 biased response. This Th2 response leads to the production of tumour-reactive antibodies. Further, neoplastic lesions and biological fluids of cancer patients contain an abundance of tumour-derived exosomes (TDE) expressing tumour antigens. Expression of tumour antigens on TDE may represent an antibody target and serve to block antibody binding to the tumour, implicating a role for these nanovesicles in tumour survival. In this study, ovarian tumour cell proteins were separated by two-dimensional electrophoresis (2-DE) and patient-derived antibodies were used to analyse immunoreactivity. Common immunoreactive proteins among ovarian cancer patients were identified by mass spectrometry and six proteins were selected based on recognition and correlation with cancer pathogenesis. The identity of these proteins were confirmed by immunoreactivity of patient-derived antibodies with recombinant proteins and their presence on in vivo and in vitro-derived ovarian tumour exosomes was defined. Analysis of the TDE demonstrated bound tumour-reactive immunoglobulins, exhibiting immunoreactivity with specific antigens, suggesting that patient-derived antibodies recognize tumour antigens on circulating exosomes.

Keywords Autoantibodies, Ovarian , Cancer, Exosomes

1. Introduction

In the tumour microenvironment, antigens are functionally and/or structurally altered by overexpression, mutation, aberrant degradation or post-translational modification that makes them immunogenic [1]. In an effective immune response, these immunogenic proteins are detected and an antitumour response is promoted to eliminate the transformed precursors before they establish malignancy. Effective elimination is characterized by the simultaneous collaboration of innate and adaptive cell-mediated and humoral responses. In the adaptive antitumour response, T cells (with cognate TCR) recognize tumour-associated antigens processed/presented on the MHC of antigen-presenting cells (APC), along with subsequent costimulation and cytokine expression for facilitation and maintenance of the response [2]. Elimina-

tion of the tumour is accomplished through the activation of cytotoxic T (CTL) cells to induce tumour cell apoptosis, activation of $CD4^+$T cells to promote both cellular and humoral responses through stimulation of APC presentation of antigens to CTL and activation of B cells to produce antigen-specific antibodies that enhance tumour cell uptake by APCs [3,4]. The primary antitumour response is facilitated by the cellular arm of the adaptive immune system; however, humoral responses to tumour antigens are clearly demonstrated through the production of antitumour antibodies [5,6]. This production of antibodies is presented as elevated IgG in the blood [7]. In ovarian cancer sera, levels of tumour reactive-IgG are elevated [8], suggesting intact humoral immunity in ovarian cancer patients and an effective humoral antitumour response. However, in the midst of this IgG-laden environment, ovarian tumours continue to thrive.

A key factor in the progression of transformed cells to malignancy is the tumour microenvironment. The tumour microenvironment consists of a number of cellular participants, including immune cells, which are critical for the suppression of tumour growth [9]. However, the functional activities of these immune cells are often counter-regulated by tumour cell expression and the release of a number of biologic components, which act to promote the growth and metastatic progression of the tumour [10]. One essential biologic component in growth and progression is the tumour-derived exosome (TDE). Studies indicate that increased release of exosomes facilitate communication between the tumour's microenvironment and the tumour cell [11]. TDE express tumour-derived antigens; however, they are not molecular duplicates of the plasma membrane of their parental tumour cells; rather, they represent a 'micromap' that displays increased expression of antigens associated with the tumour [12,13]. TDE are abundantly found in plasma and malignant effusions derived from cancer patients [14] and their presence and expression of tumour-related antigens has been documented to contribute to tumour progression. Progressive effects mediated by TDE have been found to range from regulation of tumour growth to invasion, angiogenesis and metastasis [14,15] through expression of molecules such as matrix metalloproteinases (MMP-2, MMP-9) and horizontal transfer of growth factor receptors (EGFRvIII)[16,17]. Additionally, TDE have been shown to directly and indirectly modulate the evasion of anti-tumour responses provided by effector T cells for assisting in progression. Melanoma-derived exosomes have been shown to promote monocyte production of myeloid-derived suppressor cells [14], which can act to suppress T-cell responses. Ovarian TDE demonstrate induced apoptosis of T cells by enhanced expression of Fas L on the exosomes and CD3zeta suppression on the T cell [18], while nasopharyngeal TDE have been shown to express increased galectin-9 to induce T cell apoptosis via Tim-3 [19].

Since TDE can mediate the progression of the tumour and its evasion of cellular antitumour responses, they may also intervene in the humoral antitumour response, thereby permitting further development of the tumour. Utilizing these circulating antitumour antibodies to identify immunoreactive antigens may define proteins that are essential to carcinogenesis [20]. Likewise, analyses of antigens associated with TDE may unlock the mysteries of the essential, but inadequate, humoral response needed for efficient eradication of the tumour and the role of the TDE in tumour progression and the humoral response. We hypothesize that the circulating free and TDE-associated humoral immune response can be used to identify specific tumour-associated antigens, which in the context of TDE may be a mechanism for diverting the immune response from the tumour. Using human ovarian cancer as our model system, we investigated immunoreactivity of tumour-associated antigen of ovarian patient cancer cells, patient *in vivo* and *in vitro*-derived tumour exosome antigens and patient-derived free and exosome-associated antibodies. We established that ovarian tumour-derived exosomes expressed tumour-associated antigens, which are recognized by tumour-reactive antibodies, thereby indicating a role for TDE in the ovarian tumour humoral response.

2. Materials and Methods

2.1 Patient sera, ascites and cell lines

Biofluids (sera or ascites) used in this study were derived from patients diagnosed with Stage III (T3c) serous adenocarcinoma (designated UL-124, UL-167, UL-190, UL-207, UL-351, UL-398), mucinous adenocarcinoma (UL-324), carcinosarcoma (designated UL-224), teratoma (designated UL-184) and serous/endometrioid mixed carcinoma (designated UL-472). Samples were obtained from the Gynecologic Oncology Repository at the University of Louisville, under an approved IRB protocol. Additional information about ascites sample UL-309 was unavailable. Control sera were derived from age-matched women with no evidence of ovarian disease. Primary ovarian tumour cell cultures were established from UL-124 ascites. Primary cultures were grown in 75 cm^2 tissue culture flasks, initially in Hyclone RPMI 1640 medium (ThermoScientific, Pittsburgh, PA) supplemented with 2mM L-glutamine, 10% foetal bovine serum (FBS, Biowest), 1mM sodium pyruvate (CellGro, Mediatech, Manassas, VA), 0.1 mM nonessential amino acids (CellGro, Mediatech) and 100 units/mL penicillin-streptomycin (Gibco, Life Technologies, Carlsbad, CA) in a humidified incubator at 37ºC with 5% CO_2. Cells were transferred into Ultraculture General Purpose Serum-Free Media without L-glutamine, but supplemented with the same amount of sodium pyruvate, non-essential amino acids and penicillin-streptomycin. Cell viability was evaluated by trypan blue exclusion and all cultures utilized were >95% viable.

2.2 Preparation of tumour cell lysates

Prior to the harvesting of cells, culture media was removed and saved for subsequent culture exosome isolation. Cells were rinsed twice with cold Hyclone DPBS/Modified

(ThermoScientific) and were solubilized with RIPA Buffer (1x, Pierce Chemical Co., Rockford IL) supplemented with protease inhibitor cocktail III (100x, RPI) and phosphatase inhibitor II (100x, Sigma Chemical Co., St. Louis, MO). RIPA-solubilized cells were kept on ice for 5-10 minutes and then cells were collected using a cell scraper. Solubilized cell lysate was collected into 1.5 mL microcentrifuge tubes (Eppendorf) and centrifuged at 14,000 x g for 15 minutes at 4ºC to pellet the cell debris. The resulting supernatant was collected and assayed for total protein by the Bradford assay (Bio-Rad Laboratories, Herceles, CA) using bovine serum albumin (BSA) as a standard.

2.3 Isolation of cellular protein by Rotofor fractionation

Cellular lysate was desalted by column chromatography using P6 Desalting Gel (BioRad Laboratories) and the resulting protein was assayed by the Bradford method. Desalted protein concentration was used to determine the amount of ampholyte to combine with the protein sample. Desalted protein was combined with 0.5% Bio-Lyte 3/10 Ampholyte (Bio-Rad Laboratories) and ddH_2O and loaded onto the Rotofor Cell (BioRad). Protein was focused for 4 h using the PowerPacHV under recirculated refrigeration. Focused proteins were collected into 20 fractions. The pH and protein concentrations were determined for each fraction. Each fraction (50μg) was loaded onto a Criterion Tris HCl 4-15% gel, electrophoretically separated and silver stained or analysed by western immunoblotting. Membranes were probed overnight at 4ºC with patient sera (1:250) followed by peroxidase-conjugated anti-human IgGAM (1:4000, Sigma Chemical Co). Bound immune complexes were visualized by ImmunoStar HRP chemiluminescence (BioRad) and the resulting film was analysed for similarity in reactivity between different sera. Thirteen bands corresponding to shared immunoreactivity were cut from of the Rotofor fractionated-silver stained gel and analysed by mass spectrometry (Vanderbilt University, Nashville, TN).

2.4 Protein identification by mass spectrometry

Silver-stained protein gel bands were thoroughly rinsed with water to remove residual acetic acid. Fresh reducing reagents (30 mM of potassium ferricyanide, 100 mM of sodium thiosulfate) in a 1:1 ratio were mixed and immediately added in sufficient volume to cover the gel piece. The gel slice was washed with HPLC-grade H_2O and 100 mM of NH_4HCO_3, alternatively, until the gel piece was clear. Each gel slice was diced into small pieces (1 mm^2) and placed into a clean tube, then washed twice with 100 μl (or enough to cover the gel pieces) of 25 mM NH_4HCO_3/50% ACN with vortexing for 10 minutes. The gel pieces were completely dried by Speedvac. Dried gel pieces were partially rehydrated with 5 μl of 100 mM NH_4HCO_3 and the proteins reduced in 25 μl of 10 mM DTT in 25 mM NH_4HCO_3 for 1 hr at 56ºC. Supernatant was removed and discarded and proteins were alkylated with 25 μl of 55 mM iodoacetamide in 25 mM NH_4HCO_3 in the dark for 45 minutes at room temperature. Gel pieces were washed twice in 100 μl of 100 mM NH_4HCO_3 (with vortexing for 10 minutes), dehydrated with 100 μl of 25mM NH_4HCO_3 in 50% ACN (with vortexing for 5 minutes, repeated once) and were completely dried by Speedvac for 20 minutes. Dried gel pieces were rehydrated with 5 μl of trypsin solution (0.1 μg/μl trypsin in 100 mM NH_4HCO_3) and 50-70 μl of 100 mM NH_4HCO_3 was added until the gel pieces returned to their original size. Digestion was carried out overnight at 37ºC and then stopped by the addition of 1.5 μl of trifluoroacetic acid. The resulting peptides were recovered by two-25 minute extractions using 30 μl of 50% ACN/5% formic acid at room temperature with 20 minute vortex/5 minute sonication cycles. The extracts were combined and dried in a Speedvac. The tryptic peptides were reconstituted in 25 μl of HPLC water with 0.1% formic acid.

Digested peptides were separated and mass analysed using an Eksigent Nano-LC system connected to a LTQ Velos (Thermo Fisher Scientific) ion trap mass spectrometer. Briefly, tryptic peptides were loaded onto a 150 μm ID microcapillary fused silica pre-column, which was in-house packed with 4 cm ξ 5 μm C18 resin (Jupiter C18, 5μm particle size, 300Å pore size). The C18 trap column was coupled to a nanoflow analytical column packed with 10 cm of 3 μm C18 reverse-phase resin (Jupiter C18, 3μm particle size, 300 Å pore size) constructed with an integrated electrospray emitter tip. Peptides were eluted with a 90 minute gradient (from 2%ACN with 0.1% FA to 35% ACN with 0.1% FA) over 35 minutes, followed by 35% ACN to 90% ACN over 15 minutes at a flow rate of 0.5 μl per minute. The LTQ Velos mass spectrometer was operated in data-dependent mode in which an initial MS scan recorded the mass-to-charge range as 300-2000u and the five most abundant ions were selected for subsequent collision-induced dissociation. Dynamic exclusion (repeat count 1, exclusion list size 150 and exclusion duration 60s) was enabled to allow for the detection of less abundant ions.

LC-MS/MS raw files were converted into.dta files by a custom ScanSifter algorithm. Spectra that contained fewer than 25 peaks or that had less than 2e1 measured total ion current were removed. DTA files for singly charged precursor ions were created if 90% of the total ion current occurred below the precursor ion m/z ratio and all other spectra were processed for doubly and triply charged ions. DTA files were searched against the human protein database Uniprot-human 155_200907_rev with 20,914 total protein entries using the SEQUEST algorithm. The search parameters used allowed for the following differential modifications:+57 on cysteine and+16 on methionine. SEQUEST-searched files (pepXML) were imported into ID Picker software for protein assembly. Results were filtered using the following criteria: a minimum peptide length of five amino acids, a minimum of one unique peptide per protein (modifications to cysteines or methionines were not considered distinct from the unmodified peptides), overall maximum false positive rate (FDR) of 5%, a minimum of

two additional peptides to establish a unique protein group, one protein reported per protein group and single protein groups indicated by proteins that shared the same set of peptides (indiscernible from each other based on available data).

2.5 Verification of mass spectrometry-identified ovarian cancer antigen by immunoprecipitation and immunoblot

To verify the presence of the ovarian cancer antigen identified by mass spectrometry, immunoprecipitation was performed using Protein G HP SpinTrap columns (GE Healthcare, Piscataway, NJ) and True Blot anti-rabbit or anti-mouse Ig IP Beads (eBioscience, San Diego, CA). UL-124 cell lysates were preclarified by combining 500μg lysate proteins with anti-rabbit/anti-mouse Ig IP beads. Lysate and beads were incubated at 4ºC for 1 hour, centrifuged to obtain the precleared lysate and protein concentrations of clarified lysate determined by the Bradford method (Bio-Rad Laboratories). For SpinTrap column-based immunoprecipitation, commercial antibodies corresponding to the MS-identified antigens were conjugated to the Protein G SpinTrap matrix (20μg protein/column) for 3-4 hours at room temperature and the manufacturer's cross-link protocol was followed. The antibodies used included GRP-78 (rabbit polyclonal), annexin 2 (mouse monoclonal), Cathepsin D (mouse monoclonal), alpha-enolase (mouse monoclonal), HSC 70 (mouse monoclonal) and PDI (mouse monoclonal). All precipitating antibodies were obtained from Santa Cruz Biotechnology (SCBT, Santa Cruz, CA). The unbound antibody was removed and precleared lysate in Tris-buffered saline (TBS) was added to the immunoaffinity beads and incubated overnight at 4ºC. Bound antigens were eluted using 0.1M glycine/urea as an elution buffer and the fractions were neutralized by addition of 1M Tris base. To determine the quantity of eluted protein, the Bradford protein assay was performed. For dot blot analysis, 5μl (0.5μg) of each antigen sample (in duplicate) were spotted onto individual nitrocellulose membranes (Bio-Rad Laboratories) with BSA (1 mg/mL) and HeLa or A431 (epidermoid carcinoma cell line) lysates added as negative and positive controls, respectively. Membranes were blocked in 5% nonfat milk/TBST, washed with 1xTBST (TBS +0.1% Tween-20) and incubated at 4ºC overnight with corresponding commercial antibodies (diluted 1:1000) and control or ovarian patient sera (diluted 1:250). Secondary HRP goat anti-mouse/anti-rabbit (1:5000, Bio-Rad Laboratories) or peroxidase-conjugated anti-human IgGAM (1:4000, Sigma Chemical Co) was added and immunoreactivity analysed by Immune Star HRP chemiluminescence (Bio-Rad Laboratories).

For immunoprecipitation with the TrueBlot immunoprecipitation system (Pierce Chemical Co.), 5 μg of each of the antibodies listed above were added to the precleared lysate and incubated for three hours at 4ºC. The incubated GRP-78/lysate sample was added to the True Blot anti-rabbit beads, with the remaining Ab/lysate samples added to the True Blot anti-mouse beads for overnight incubation at 4ºC. Following this incubation, supernatants were collected by centrifugation at 10,000xg; beads were washed 3x in a cold NP-40 Lysis Buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1% NP-40) and supernatant aspirated, and beads were combined with 50 μL of 1x Laemmli. The bead complexes were boiled and centrifuged to collect supernatant containing the immunoprecipitated antigen.

The immunoprecipitated antigen samples were loaded onto Mini-Protean TGX Precast Gels (4-15%, Bio-Rad Laboratories), electrophoretically separated and analysed by western immunoblotting. Membranes were blocked and probed overnight at 4ºC with corresponding commercial antibodies, as previously mentioned. Secondary rabbit and mouse TrueBlot anti-rabbit/anti-mouse IgG HRP (eBioscience) were added, followed by analysis of immunoreactivity using Immune Star HRP chemiluminescence (Bio-Rad Laboratories).

2.6 Recognition of recombinant proteins by ovarian cancer sera antibodies

To verify recognition of the mass spectrometry-identified proteins by ovarian cancer sera, 0.2 μg human recombinant protein for GRP-78 (ProSpec Bio, East Brunswick, NJ), annexin 2 (ProSpec Bio), cathepsin D (ProSpec Bio), PDI (ProSpec Bio), alpha-enolase (American Research Products, Waltham, MA) and HSC70 (Enzo Life Sciences, Farmingdale, NY) were combined with 4x LDS nonreducing buffer (ThermoScientific), loaded onto a 10% SDS-PAGE gel and analysed by western immunoblotting using serum from a non-cancer control (Ctrl, 1:250) and ovarian cancer patients (samples UL167, UL190, UL207, UL324, UL351, UL398). All ovarian sera were used at 1:250, with the exception of the mucinous sample (1:100), based on a lack of detection at the greater dilution. In addition, as a positive control, the recombinant proteins were probed with a mixture of commercial antibodies against the identified proteins (1:1000) to further ensure detection of the recombinant antigen.

2.7 Isolation of ovarian tumour exosomes and analysis of tumour-exosome protein immunoreactivity

Exosomes were isolated from ovarian tumour-patient (UL-124) ascites and conditioned ovarian tumour culture media by a two-step chromatographic procedure developed in our laboratory [18]. Ascites and media, either serum-containing or serum-free, (100 mL), were concentrated by ultrafiltration and applied to a 2% agarose (Agarose Bead Technologies, Tampa, FL) chromatography column (1.5 x 20 cm) and equilibrated with ddH_2O. Fractions (1 mL) were collected and the elution was monitored by absorbance at 280 nm. Fractions containing material greater than 50 million Daltons were obtained and ultrafiltrated to a concentrate, and the quantity of protein

was assayed using the Bradford method. The biofluid-derived vesicular proteins (15µg) were mixed with a 1x Laemmli sample buffer, loaded on a 10% SDS-PAGE gel and analysed using western immunoblotting. Immunoreactivity of tumour-exosome-derived protein was assayed by commercial antibodies corresponding to mass spectrometry-identified proteins.

2.8 Isolation of IgG from TDE and analysisof immunoreactivity

1 mL of the UL-124 ascites bound exosome fraction was combined with 50µL increments of the IgG elution buffer until a pH of ~3.0 was obtained. The sample was allowed to incubate with end-over-end rotation for 30 minutes at room temperature. Post incubation, the sample was added to a pre-rinsed (1x PBS) Vivaspin 2 centrifugal concentrator with a membrane MWCO of 1 x 10^6 (Sartorius Stedim Biotech, Bohemia, NY). IgG was eluted from the exosomes by centrifugation (4000 x g, 10-20 min.) and collected from the filtrate tube. IgG was neutralized using 1M Tris-HCl, pH 9.0 and protein quantitated using the Bradford method. 0.5µg Human recombinant protein corresponding to the antigens of interest was loaded onto a Mini PROTEAN TGX 4-15% gel and western immunoblot performed using the isolated IgG at 1:50 to detect immunoreactivity.

3. Results

3.1 Recognition of ovarian tumour antigens by patient serum antibodies

Previously, traditional isoelectric focusing of ovarian tumour antigenic proteins evaluated by silver staining of IPG SDS-PAGE gels containing focused protein followed by immunoblotting with serum-derived antibodies from ovarian carcinosarcoma patient (UL-224) and an ovarian teratoma patient (UL-184) revealed distinct patterns of immunoreactivity associated with cancer patients. Subsequently, Rotofor fractionation has been used to fractionate cellular proteins based on isoelectric focusing. Ovarian tumour antigenic proteins were initially Rotofor fractionated, this material was then separated by SDS-PAGE gels and subsequently immunoblotted with sera from patient UL-224 (Figure 1a) and UL-184 (Figure 1b) as primary antibodies. Bands corresponding to shared recognition (n=13) between sera were subjected to mass spectrometry. Similar reactivity between patient-derived antibodies to antigenic proteins was noted with some variation in intensity (for example, stronger recognition with UL-224: fraction 12; stronger recognition with UL-184: fraction 9, lower band).

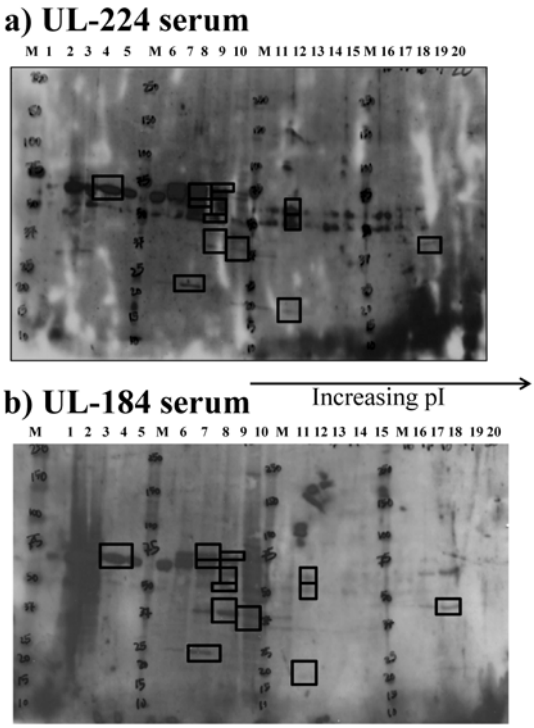

Figure 1. Shared immunoreactivity of Rotofor-fractionated UL-124 serum-free lysate with serum of ovarian tumour-patient a) UL-224 (1:250) and b) UL-184 (1:250). Boxes indicate randomly chosen bands' shared reactivity. Molecular marker (MM) and Rotofor fraction numbers (1-20) are indicated above each image. Increasing isoelectric point (pI) is indicated by the arrow beneath images.

3.2 Identification of ovarian tumour antigens by patient antibodies

From the 13 selected bands, mass spectrometry analysis identified six proteins with high confidence: GRP 78 (glucose-related protein 78), annexin 2, cathepsin D, alpha-enolase, HSC70 and PDI (protein disulfide isomerase). These were selected for focus due to their protein scores, their association with tumour survival and their status as a potential biomarker. In order to verify the presence of these antigenic proteins in the ovarian cancer lysate sample, these proteins were isolated from tumour cell lysates by immunoprecipitation. The proteins of interest were subjected to western and dot immunoblotting and probing with commercial antibodies corresponding to the antigens of interest. For the dot blot assays, captured antigens were applied to the membrane in duplicate. Immunoreactivity of precipitated antigen for all antigens of interest was detected by dot blot (Figure 2A). In some cases, the presence of specific proteins was verified by western immunoblotting (Figure 2B); however, some proteins failed to demonstrate reactivity in western immunoblotting. This was potentially due to the loss of specific epitopes through protein reduction and heating.

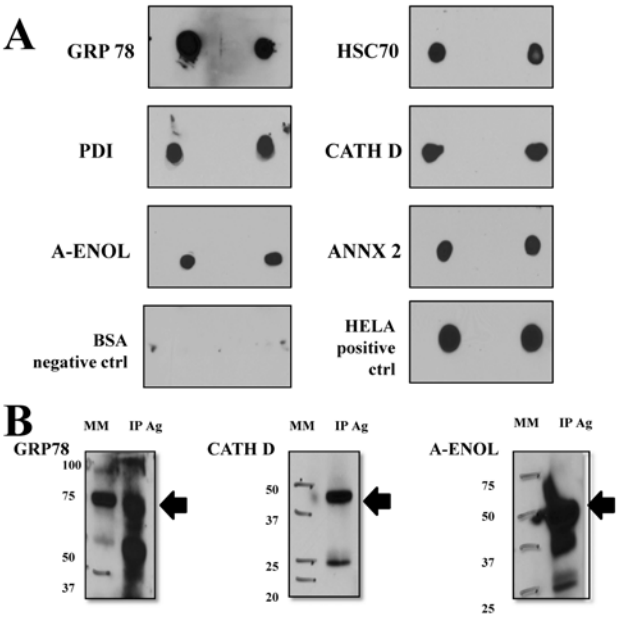

Figure 2. Representative immunoblots validate the presence of mass spectrometry antigens of interest using commercial antibodies. UL-124 cellular antigen was immunoprecipitated by a) Protein G HP SpinTrap columns, applied in duplicate and subjected to dot blot; and b) True Blot IP beads subjected to western blot (representative immunoreactivity shown).

3.3 Validation of serum antibody detection of mass spectrometry antigens of interest using corresponding recombinant proteins

Humoral responses detecting GRP 78, annexin 2, cathepsin D, alpha-enolase, HSC70 and PDI were evaluated by western immunoblot using recombinant proteins and sera from patients with serous adenocarcinoma/carcinoma and mucinous adenocarcinoma. Using patient-derived antibodies (Figure 3a-e), western immunoblotting identified the presence of annexin 2, alpha-enolase and HSC70 with variations in intensities and isoforms between samples. Preliminary assays for antigen detection by serum revealed minimal detection of Cathepsin D by the serous samples tested, with the exception of patient UL-351. Out of the serous carcinoma samples assayed, patient 351 displayed lone recognition of the intermediate Cathepsin D isoform (~46 kDa). Additionally, reactivity to isoforms of HSC70 (~30 kDa) and annexin 2 (~75-90 kDa) were detected to varying degrees by the serous samples. Mucinous adenocarcinoma patient antibodies (Figure 3f) detected GRP78 and alpha-enolase, and faintly detected the intermediate form of Cathepsin D (~46 kDa). Additionally, the higher molecular weight isoform was detected with annexin 2, as seen in some of the serous samples. A mixture of commercial antibodies (Figure 3g) corresponding to recombinant proteins of interest was utilized to detect viability of the antibodies and their ability to recognize the antigens at their standard molecular weights. Of the six recombinant proteins assayed, the commercial antibodies recognized GRP78, annexin 2 (both isoforms), alpha-enolase and PDI. Identification of recombinant antigens using serum antibodies from normal, non-cancer presenting controls (Figure 3h) similarly recognized alpha-enolase and HSC70 at its standard molecular weight and the lower molecular weight form.

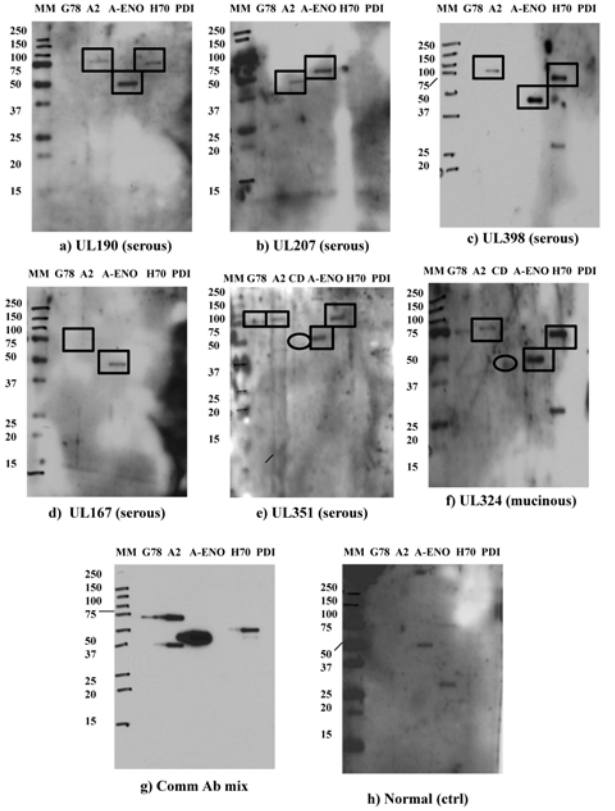

Figure 3. Representative immunoblot validating ovarian cancer patient antibody recognition of antigens of interest. Immunoreactivity of human recombinant protein with human ovarian serum from patients with: a-b) serous adenocarcinoma; c-d) serous carcinoma; d) mucinous adenocarcinoma; f) commercial Ab mix; g) non-cancer control (Ctrl). Recombinant proteins indicated as: G78 (GRP78), A2 (annexin 2), A-eno (alpha-enolase), H70 (HSC70), PDI (protein disulfide isomerase) and CD (Cathepsin D), were shown with serous sample 351, mucinous sample and control. Ovals indicate recognition of CD. Boxes indicate recognition of other antigens. Molecular marker indicated by MM. Initial assays showed no reactivity of CD with other sera samples.

3.4 Enrichment of ovarian tumour mass spectrometry antigens of interest in in vivo and in vitro-derived ovarian tumour exosomes

Previous work conducted by our group demonstrated the presence of bound immunoglobulins on circulating exosomes. These exosome-bound antibodies appear to recognize specific proteins. To determine whether ovarian tumour-derived exosomes are enriched with the same antigens identified by mass spectrometry of ovarian tumour cells, exosomes from UL-124 ascites or cell culture were probed in western blots with commercial antibodies against the antigens of interest from the mass spectrometry analysis. Western immunoblot analysis of ascites-derived and culture-derived exosomes demonstrated the presence of GRP 78, alpha-enolase, PDI and varying isoforms of Cathepsin D (Figure 4).

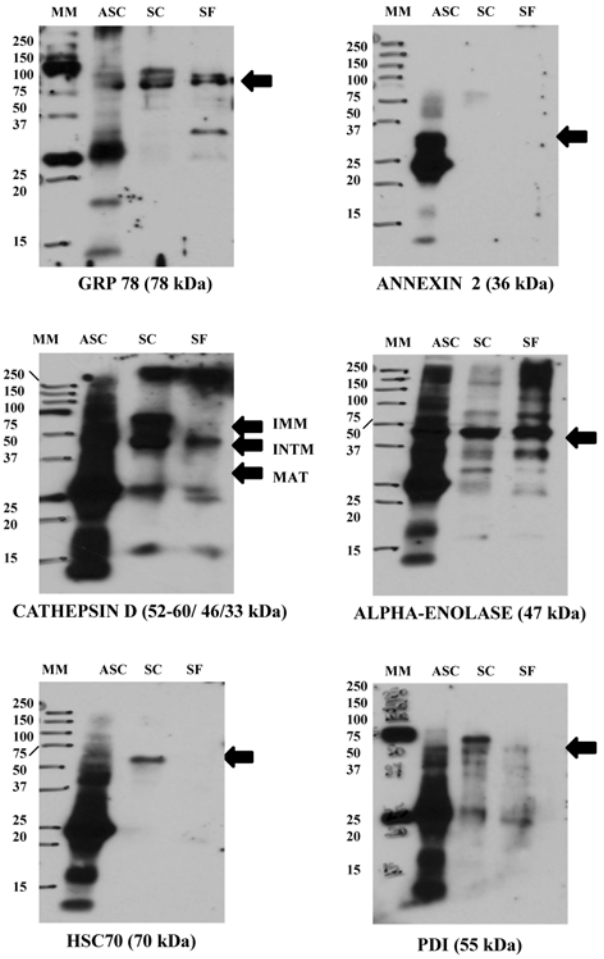

Figure 4. Analysis of the association of mass spectrometry antigens of interest with *in vivo* and *in vitro*-derived exosomes. Western immunoblots of UL-124 ascites-derived (ASC), serum-containing (SC) and serum-free (SF) culture-derived exosomes demonstrating immunoreactivity to commercial antibodies corresponding to the antigens of interest. Cathepsin D isoforms: immature (IMM), intermediate (INTM), mature (MAT).

The presence of the Cathepsin D active intermediate enzyme (~46 kDa) was detected in all samples, while the immature proenzyme (52-60 kDa) was detected in ascites and culture media. The active mature form (~33 kDa) was detected in ascites only. Variation in recognition was seen between the culture-derived samples, particularly in the identification of annexin 2 and HSC70 in FBS-containing vesicles and whose expression was shared in the ascites sample (Figure 4). Since cells grown in serum-free media are more sensitive to factors such as pH, temperature, mechanical stresses, etc., the molecular expression of specific proteins may have been altered.

3.5 Exosome-bound immunoglobulin reactivity to mass spectrometry antigens of interest

To examine the ability of exosome-associated IgG to recognize the antigens of interest, bound-immunoglobulins were eluted from exosomes isolated from the ascites fluid of patient UL-124. Recombinant proteins corresponding to the antigens of interest were subjected to electrophoresis and their immunoreactivity was examined with the isolated exosome-associated antibodies (Figure 5). Exosome-associated immunoglobulins exhibited recognition of both isoforms of annexin 2, alpha-enolase and HSC70, with minor recognition of GRP 78.

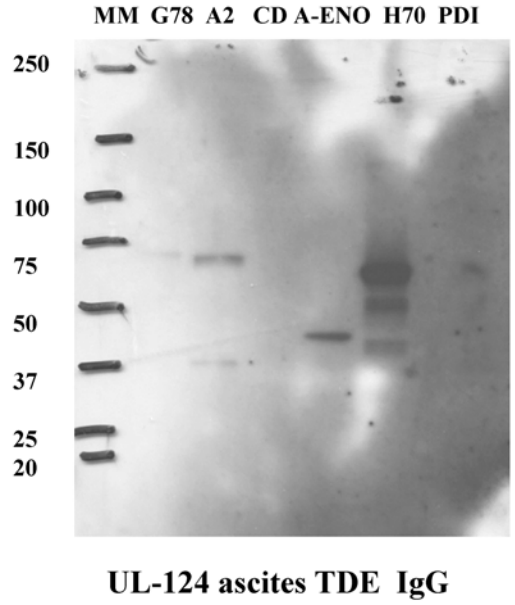

Figure 5. Recognition of antigens of interest by patient ascites exosome-associated IgG. Western immunoblot of recombinant proteins' immunoreactivity to UL-124s ascites exosome-associated IgG. Molecular marker indicated by MM. Recombinant proteins indicated as in previous figures.

4. Discussion

Cellular proteins present in the tumour microenvironment can be functionally and/or structurally altered, resulting in them becoming immunogenic and provoking autoantibody responses. These autoantibodies reflect responses to tumour-associated (differentially expressed on normal and cancer cells) antigens and can be detected in the circulation regardless of the magnitude of tumour-associated antigen expression or tumour size [21]. As a result of the long-lived humoral response, the antigen presence is "amplified," which facilitates ease of detection. Since autoantibodies act as sentinels of aberrant cellular activity and their presence reflects the execution of a humoral immune response to the tumour, detailed analysis of their immunoreactivity can provide a clearer understanding of the tumour-associated antigens that they recognize [22].

Progress in the characterization of human cancers has been attained through proteomic analyses of cancer-associated serum proteins. Proteomic analysis involves extensive characterization of proteins, including identification of any modifications and interactions, and structural determination of isoforms and related functional components. Traditional approaches to quantitative analysis of proteomes involve high resolution separation of the proteins (by size and charge) using 2DE, accompanied by identifi-

cation of the resolved proteins using mass spectrometry (MS) [23]. Protein profiles from such analyses can reveal differential protein expression between samples, which may denote key molecules that are critical to protein function.

In this investigation of ovarian cancer patients' humoral antitumour responses, we used patient serum autoantibodies for the detection of ovarian-cancer-specific-antigenic proteins. Proteomic analysis allowed for the identification of numerous proteins with shared recognition: six of particular interest (GRP78, annexin 2, Cathepsin D, alpha-enolase, HSC70 and PDI), due to their roles in tumour survival and as potential biomarkers for different cancers. In this instance, the identities of these antigenic proteins were confirmed by western immunoblotting and dot immunoblotting of immunoprecipitated ovarian tumour cell-derived proteins, using commercial antibodies against these proteins. One concern in this study was the effects of denaturation on the ability of the antigenic protein to be recognized by our commercial antibodies. Hence, we utilized both western and dot immunoblot analysis to reduce the likelihood of "overlooking" the detection of the antigens. As expected, there was variability in detection of annexin 2, HSC70, and PDI between western and dot blot analysis. Dot blot analysis revealed detection of all antigens, while western blot resulted in definitive detection of GRP 78, Cathepsin D and alpha-enolase.

GRP78, a member of the heat shock protein 70 family, is a chaperone protein commonly housed in the lumen of the endoplasmic reticulum, but is also expressed on the cell surface in various cancers [24]. Cell-surface expression of GRP78 in cancer cells has been shown to activate pathways that induce cellular survival and proliferation [24], and to correlate with the expression of circulating autoantibodies in prostate [25] and ovarian [26] cancers. Previous studies of circulating ovarian cancer sera autoantibodies by this lab demonstrated recognition of GRP78 and Cathepsin D (immature proenzyme form and mature form) in ovarian cancer patients. In this study, immunoprecipitated antigen probed with commercial antibodies revealed bands ~75 kDa (corresponding to GRP78), ~48 kDa (corresponding to the intermediate form of Cathepsin D) and ~47 kDa (corresponding to alpha-enolase). Cathepsin D is an aspartic lysosomal peptidase present in normal cells, but its overexpression has been reported in a number of cancers [27]. Functionally, it is believed to act on tumour growth and metastasis by assisting with stromal remodelling. Processing of the single polypeptide immature (pro) form (52 kDa) of Cathepsin D involves removal of the propeptide to form the active intermediate isoform (48 kDa) and eventual formation of the double-chained mature isoform (34 kDa heavy chain, 14 kDa light chain). Alpha-enolase is a glycolytic enzyme important in the catalysis of 2-phospho-D-glycerate (PGA) to phosphoenolpyruvate [28], and is upregulated under stressful conditions, like hypoxia, in order to mediate enhanced anaerobic metabolism. Increased autoantibodies against alpha-enolase have been recorded in some cases of organ-specific autoimmunity [29] and in a number of metastatic cell lines. This study indicates strong recognition of immunoprecipitated ovarian cancer antigens by commercial anti-Cathepsin D and alpha-enolase antibodies. Consistent recognition of human recombinant alpha-enolase by serous adenocarcinoma/carcinoma and mucinous adenocarcinoma serum antibodies was seen, with variation in Cathepsin D and GRP78 recognition between sera types. Additionally, recombinant alpha-enolase also appeared to be weakly recognized by non-cancer controls. GRP78 and alpha-enolase were also detected in all 3 exosome samples, with detection of all 3 isotypes of Cathepsin D among the exosome samples. Exosome-associated antibodies also recognized alpha-enolase with faint detection of GRP78. Our data suggests that GRP78, Cathepsin D and alpha-enolase are tumour-associated antigens that are transported to the released exosome and elicit a humoral response, indicated by the presence of antibodies to these molecules.

Annexins are calcium-binding proteins with functions ranging from trafficking of vesicles, to apoptosis and regulation of cellular growth [29]. Annexin 2, a key mediator in the plasminogen activator system, has been shown to be important in clot dissolution and wound healing, and integral to cancer progression. Located on the surface of endothelial cells and various tumour cells, annexin 2 can exist as a monomer (~36 kDa) or a heterotetramer (~90 kDa), which acts a binding protein for procathepsin B on the surface of tumour cells to facilitate invasion and metastasis. Expression of annexin 2 is varied in different types of cancers with increased expression in pancreatic [30] and breast [31] cancers, but variable expression in prostate cancer [32]. In our studies, the presence of annexin 2 was validated by dot blot and ovarian cancer patient serum antibodies primarily recognized the heterotetramer form of the antigen, while both isoforms were detected in the ascites TDE. Furthermore, IgG isolated from TDE detected both isoforms.

Chaperone proteins HSC70 and PDI were detected by dot blot assay and were also found to be associated with TDE. HSC70 is a constitutively expressed cytoplasmic protein that binds to new peptides exiting the ribosomes to protect the hydrophobic residues from inefficient interactions. Additionally, it can catalyse the disassembly of clathrin cages and has been implicated in the regulation of tumourigenesis and apoptosis. With the assistance of co-chaperones, it can be recruited to the intracellular membrane, but can be released from cells as a result of active secretion [33]. Studies of a cell line transfected with Cathepsin D showed that overexpression of Cathepsin D resulted in an increase in the malignant phenotype of the transformed cells [34]. Subsequent studies of these cells have revealed that this overexpression prevented the release of HSC70, which led to the malignant phenotype. In our study, the expression of Cathepsin D was seen in the ovarian cancer cells and all

isoforms on the exosomes. However, the ovarian patient serum antibodies failed to detect Cathepsin D in all cases. The antigen expression of Cathepsin D, the lack of antibodies to cathepsin D, and the reduced HSC70 expression on the cells may indicate a "protective" mechanism used by the tumour in order to maintain high Cathepsin D expression, so that HSC70 release can be minimized and the tumour growth can be maintained. Furthermore, the exosome expression of all three isoforms may act to "block" recognition of antigens on the tumour cell, thereby protecting the tumour from immunosurveillance. PDI is a key endoplasmic reticulum chaperone that catalyses the breakage of disulfide bonds within a protein and rearranges them to form the native protein. Because accumulation of misfolded proteins in the cell can lead to enhanced cellular stress and death [35], upregulation of PDI can act to reduce stress-associated apoptosis. Human PDI-family member ERp5 has been shown to promote shedding of MICA from epithelial tumours [36]. MICA is the ligand for the NKG2D (NK group member 2D) receptor on NK, $CD8^{+}$T, NKT and ⊙⊙T cells, and upon ligation, activates cytolysis of the target cell. Tumour-derived exosomes have been shown to express MICA/B, which downregulates NKG2D expression to reduce NKG2D-mediated killing [37]. In our studies, PDI was strongly expressed in TDE. Expression of PDI in TDE may participate in the facilitation of MICA expression, resulting in interference of innate and adaptive immune effector cell function against the tumour.

5. Conclusions

Studies of TDE have demonstrated their critical role in cancer pathogenesis; however, much remains to be discovered about their roles in the humoral antitumour response. The vast amount of autoantibodies present in the circulation clearly indicates that a humoral response is mounted against the tumour, but that its efficacy in mediating tumour elimination is compromised [38]. TDE cannot only express tumour antigens, but are also able to elicit a humoral response against the antigen, as indicated in this study. We propose that the tumour cell expresses certain antigens in their exosomes in order to divert the humoral immune response away from the tumour, thereby preventing its effective detection and elimination.

6. References

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