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Introduction

This concept award was funded to develop the use of a bacterial toxin (alpha toxin) from *C. Septicum* to capture glycosylphosphatidyltnositol anchored proteins (GPI-APs) from breast cancer tissue and serum for identification by mass spectrometry. The rationale that supports this research is that there are increases in the levels of enzymes that produce the GPI anchor likely leading to increased levels of GPI-APs on the tumor cell. The continued selection of this molecular alteration must be providing a growth advantage to tumor cells. Therefore, identification of the proteins that are GPI-anchored in breast cancer and not normal breast tissue may lead to new diagnostic and therapeutic targets for breast cancer.

Research Accomplishments

This was a one year grant designed to jump start this work and the project is still in progress. We are applying for an NCI EDRN biomarker development grant to continue this work in the future. The statement of work that was established for this project stated that we would use a cold triton X-100 technique to extract detergent resistant membrane proteins for capture by alpha toxin. This method of membrane extraction did not work optimally for breast tissue. Therefore, a substantial amount of work went into optimizing a membrane extraction technique that yields high quality MS/MS data from breast tissue. This is one of the major accomplishments of this grant is the development of a protocol that can be used for proteomic analysis of membrane proteins from breast tissues.

Another positive accomplishment of this project is the validation that the toxin is efficient at recognizing GPI-APs. To demonstrate this in a controlled manner we established breast cancer cell lines expressing a control siRNA sequence or a sequence that silences GPI-specific phospholipase D (GPI-PLD). The control cells should have less GPI-APs on the cell surface while GPI-PLD suppressed cells will have more GPI-APs on the cell surface as the release of these proteins are inhibited. We used these cells to optimize the membrane extraction method used in the study and to validate that the toxin was binding proteins in a GPI anchor-dependent manner. Data shown illustrate that the toxin binds preferentially to membrane proteins isolated from GPI-PLD siRNA expressing cells compared with control cells (Fig. 1 compare last 2 lanes). The amount of total protein that was put into the toxin capture assay was the same for both cell lines (Fig. 1, first 2 lanes). Next, we wanted to determine if the toxin could bind to circulating GPI-APs in serum and evaluate if there was a difference in the level of toxin binding between normal serum and serum from breast cancer patients. Our results are very promising. Western blot analysis using the biotinylated toxin followed by detection with streptavidin-conjugated horseradish peroxidase and ECL reagent reveal that the toxin binds more to proteins from the serum of breast cancer patients versus normal serum (Fig. 2, left panel). We then made a pool of these normal sera and breast cancer sera to test the ability of alpha toxin to bind GPI-APs in solution. Toxin coupled to magnetic beads captured proteins from a pooled sample of serum from breast cancer with minimal binding to proteins from pooled normal serum (Fig. 2, right panel). The molecular weight range of the proteins captured by alpha toxin matches the area diffusely stained using biotinylated toxin (98-62 kD range). These results suggest that toxin binding alone even without the identification of the proteins binding the toxin may be useful as a diagnostic. We have received a set of 24 serum samples that are half early stage breast cancer and half normal serum. We are blind to the sample designation and will analyze with these 2 methods, toxin overlay and toxin pull down. Once we determine results of our analysis we will compare with the identity of the unknowns. This will establish if the toxin binding alone can distinguish breast cancer from normal.

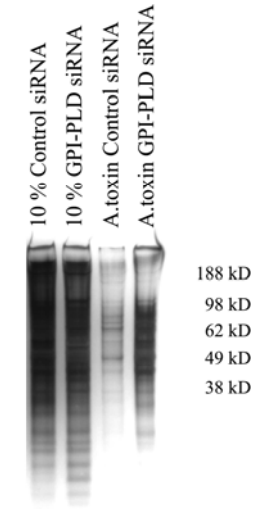

Fig. 1 Silver-stained gel of input and alpha toxin capture from membrane extracted proteins from control siRNA and GPI-PLD siRNA expressing MDAMB231 cells.

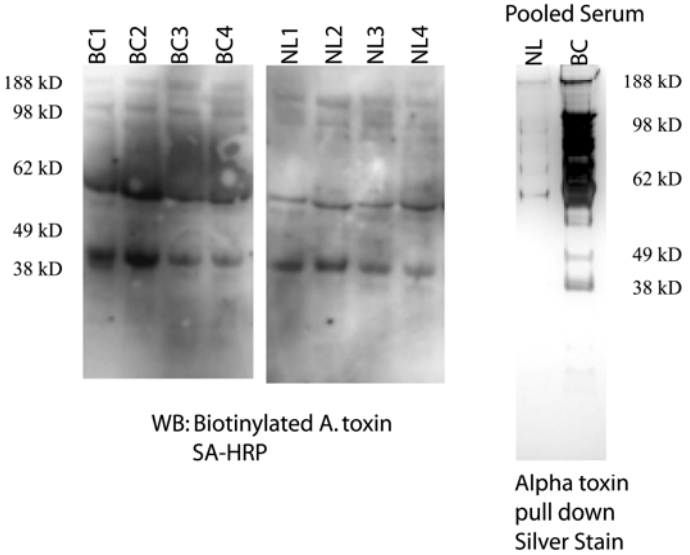

Fig. 2 Toxin overlay and toxin pull down analysis of serum samples from patients with ductal invasive breast cancer (BC1-BC4) or normal serum from healthy women (NL1-NL4). The toxin overlay is performed using blots from 1 µl of serum separated on gradient 4-12 % Bis-Tris gels. Biotinylated toxin (2 µg/ml) diluted in 5 % non-fat milk blotto is the primary. Bound toxin was detected using streptavidin-HRP followed by ECL reagent. The toxin pull down was performed using 5 µl of pooled serum normal (same samples analyzed in the overlay) or pooled serum breast cancer (same cases in toxin overlay) mixed with alpha toxin beads (10 mg/ml) for 1 hour at room temperature. Bound proteins were separated on gradient 4-12 % Bis-Tris gel prior to fixation and silver stain.

We are currently analyzing MS/MS data from aim 1 and our preliminary results are very interesting. We are finding that a large portion of

the proteins binding with the toxin are involved with control of apoptosis and cell cycle control. Many of these proteins are not predicted by databases such as GPI SOM to be GPI anchored. Therefore, it may be a possibility that these proteins are produced as spice variants or fusions that cause GPI anchoring. This type of mislocalization of a protein from intracellular to membrane can significantly alter its function. Our future experiments will focus on demonstrating that this mislocalization is occurring and validating that GPI anchoring is the reason.

We have purified toxin/DNA molecules that can be used for the RT-PCR diagnostic assay proposed in aim 2. The assay will capture proteins identified as binding to the toxin from serum followed by addition of the toxin/DNA molecule. The DNA sequence is known and can be amplified by PCR to drive the sensitivity of detection to lower levels. We have ordered antibodies to several target proteins and will begin to optimize this assay. Initial test of these antibodies using Western blot are not promising, likely because of lack of sensitivity. Next, we will evaluate the antibodies in an ELISA format so that a larger amount of serum can be used to bind with the antibody.

Key Accomplishments:

We have developed a method for membrane extraction from breast cancer tissue that works well for MS/MS.

We are analyzing MS/MS protein datasets to identify proteins of interest based on comparison of matched sets of tissue from 3 breast cancer cases before and after alpha toxin enrichment.

We have discovered that alpha toxin binding to glycoproteins in serum may be on it's own a sensitive diagnostic assay for breast cancer. These results are going into an NCI EDRN application for biomarker discovery. Access to EDRN training sets will help validate these preliminary results.

We have learned that intracellular proteins may be aberrantly receiving GPI anchors in breast cancer cells. This research can foster future projects to discover new targets for breast cancer therapy as well as diagnostic assays.

In the next year several publications will be written and we will cite the BCRP concept award.

Reportable Outcomes:

Data generated will be included in an Early Disease Research Network (EDRN) biomarker discovery lab application.

Manuscripts in the next year will be prepared and submitted citing this award.

Conclusion: The data generated by this breast cancer concept award will provide new markers and potential therapeutic targets for breast cancer in the future. The original hypothesis that grounded this application was that the amplified expression of GPI anchored proteins by breast cancer cells must produce a growth advantage for the tumor cell. Many of the novel markers currently being investigated as a result of this research have functions in the control of the cell cycle and the regulation of apoptosis. These proteins may not normally have a GPI anchor and the aberrant addition of a GPI anchor to the proteins may be turning off the regulatory role of these proteins enabling the tumor cells to proliferate and survive. Many future studies can evolve from this study (i) prove that these proteins are changing cell localization and function in breast cancer (ii) determine the use of alpha toxin as a diagnostic probe for breast cancer (iii) Determine the structures of the GPI anchor glycans in breast cancer and compare to GPI anchor glycan structures from normal cells. This information may be useful to produce vaccines against breast cancer in the future (iv) determine the minimal region of the toxin required for binding to GPI anchor glycans. This may be useful as a targeted drug delivery molecule.