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Elevated levels of glycosylphosphatidylinositol (GPI) anchored proteins in plasma from human cancers detected by *C. septicum* alpha toxin

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Abstract

The glycosylphosphatidylinositol (GPI) anchor is a glycan and lipid posttranslational modification added to proteins in the endoplasmic reticulum. Certain enzymes within the GPI biosynthetic pathway, particularly the subunits of the GPI transamidase, are elevated in various human cancers. Specific GPI anchored proteins, such as carcinoembryonic antigen and mesothelin, have been described as potential biomarkers for certain cancers; however, the overall levels of GPI anchored proteins present in plasma from cases of human cancers have not been evaluated. We have developed the use of a bacterial toxin known as alpha toxin from *Clostridium septicum* to detect GPI anchored proteins *in vitro*. In this study, we use alpha toxin to detect GPI anchored proteins present in plasma from cases of several types of human cancers. Our data indicate that human cancers with previously documented elevations of GPI transamidase subunits show increased alpha toxin binding to plasma from patients with these cancers, indicating increased levels of GPI anchored proteins. Furthermore, our results reveal very low levels of alpha toxin binding to plasma from patients with no malignant disease indicating few GPI anchored proteins are present. These data suggest that GPI anchored proteins present in plasma from these cancers represent biomarkers with potential use for cancer detection.

Keywords

Cancer; plasma; GPI-anchored proteins; GPIT; alpha toxin; human

1. Introduction

A particular glycosylation known as the glycosylphosphatidylinositol (GPI) anchor is a unique type of glycoconjugate added to certain cell surface glycoproteins that contain a C-terminal signal sequence in addition to the N-terminal signal sequence. This specialized glycan and lipid-containing membrane linkage is highly conserved in all eukaryotic species

establishing the importance of this form of glycosylation. The GPI anchor consists of a core structure that is assembled and added en bloc to the C-terminus of proteins by the GPI transamidase (GPIT) multisubunit complex. Catalytic and non-catalytic subunits of the GPIT exhibited increased expression at the mRNA and protein level in many human cancers [1–3]. For example, breast carcinoma has significant elevations in the *GPAA1* and *PIGT* subunits of the GPIT due to chromosomal amplifications. The increased expression of these non-catalytic subunits was found to significantly increase both the tumorigenicity and the overall levels of GPI anchored proteins in breast carcinoma [1,4].

The overall abundance of GPI anchored proteins is estimated to comprise 1–2% of all translated proteins in the human proteome [5]. Their abundance in specific tissues may be lower due to the regulation of specifically expressed GPI acceptor proteins. We have been utilizing alpha toxin to capture and identify GPI anchored proteins by mass spectrometry [4]. Our studies have revealed that alpha toxin binds with the GPI glycan region as shown by retained binding of the toxin after removal of the lipid portion of the GPI anchor with GPI-specific phospholipase C. Furthermore, the diversity of GPI anchored proteins that bind the toxin indicates that the binding occurs via the GPI glycan without peptide requirements. Therefore, alpha toxin can be used as a lectin specific for the GPI anchor [4].

The primary mechanism that allows GPI anchored proteins from tumors to enter the circulatory system is not well understood. GPI anchored proteins can potentially be released from cells by proteolysis [6], GPI-specific phospholipase activities [7,8], or by exosome vesicular transport from the cell membrane [6] (Fig. 1). The GPI glycan would remain attached to the GPI anchored proteins and bind with alpha toxin if the proteins were released by exosome or GPI-specific phospholipase cleavage (Fig. 1). However, GPI anchored proteins released by proteolysis would not bind alpha toxin due to lack of a GPI anchor. Our goal with the current study is to determine if alpha toxin can detect elevated levels of GPI anchored proteins present in plasma samples obtained for various human cancers.

2. Patients and methods

2.1. Patients

Blood from non-diseased and patients with ductal invasive breast carcinoma, ovarian cancer, kidney cancer, colon cancer, liver cancer, lung cancer, or brain cancer was collected pre-operatively and in accordance with approved institutional review board human subject guidelines at Georgia Health Sciences University (MCG) or the Ovarian Cancer Institute (Table 2). Plasma fractions were stored at -70°C until use.

2.2. Slot blot and alpha toxin detection

Plasma (5 μl) was mixed with 5 μl Laemmli buffer. Samples were heated to 95°C for 2 minutes to denature the proteins and cooled to room temperature before application to the membrane. Nitrocellulose Protran BA85 membrane was immobilized and clamped securely using a Schleicher and Schuell Minifold I Slot Blot System. The samples were added to each slot before the vacuum was applied for 1 minute. Each well was washed using 1X PBS (200 μl) and again the vacuum was applied for 1 minute before removal of the membrane from the apparatus. The membrane was blocked in 5% milk/1X TBST (Blotto Solution) overnight

at 4°C. The blot was incubated with biotin labeled alpha toxin (2 µg/ml) expressed, purified, and labeled as described previously [4]. Bound toxin was detected using a 1:5,000 dilution of streptavidin-HRP (Vector Labs, Burlingame, CA) before washing and detection using Western Lightening Plus (Perkin Elmer). Slot blots were then stripped using 0.1 M glycine pH 2.9 overnight, blocked again, and detected using anti-alpha 1 acid glycoprotein antibody (Sigma) to normalize for total protein content. Intensity of alpha toxin binding was determined using ImageJ analysis and was normalized to total protein band density.

2.3. Phospholipase C treatment and detection of CEA5 in LS174T cells

Approximately 10×10^6 LS174T colon cancer cells were collected by gentle cell scraping. Cells were diluted with 200 µl 1X PBS supplemented with 5 mM calcium and magnesium chloride and evenly split into two fractions. One fraction received buffer only and one received 1.5 U/ml GPI-specific phospholipase C (Invitrogen) for 1 hour at 37°C. The cells were collected by centrifugation and the supernatants were collected for analysis. Biotin labeled alpha toxin 2 µg/ml, was added and the samples were incubated at room temperature for 30 minutes. Streptavidin magnetic beads (Promega) were added for 30 additional minutes at room temperature. Beads were captured on a magnetic stand and washed 3X with 1X PBS before releasing the proteins with Laemmli buffer. Proteins were separated on a 4–12% Bis-Tris polyacrylamide gel (Invitrogen) and transferred to PVDF for detection of CEA5 (monoclonal antibody COL-1, Invitrogen) or gels were fixed and stained with silver.

3. Results

In our previous study, we suppressed the expression of the *GPAA1* and *PIGT* subunits of the GPIT, enabling us to establish that alpha toxin binding required the addition of the GPI anchor to proteins [4]. Here, we show that alpha toxin can also bind to GPI anchored proteins that have been cleaved by GPI-specific phospholipase. As shown in Fig. 2A, LS174T colon cancer cells were incubated with or without GPI-specific phospholipase C. Biotin labeled alpha toxin was then added to the released proteins to capture GPI anchored proteins. Western blot detection of the GPI anchored protein carcinoembryonic antigen 5 (CEA5) indicate that CEA5 is present in the input supernatant samples from both untreated and PI-PLC treated cells. However, alpha toxin only captured CEA5 from the supernatant of PI-PLC treated cells indicating that the CEA5 released endogenously from LS174T cells does not contain the GPI anchor glycan and is released by proteolysis. Equivalent levels of proteins were present from the supernatants of both reactions (Fig. 2B); therefore, alpha toxin binding requires the presence of the GPI anchor glycan attached to GPI anchored proteins as found following PI-PLC release.

The levels of GPI anchored proteins present in plasma can be controlled by many factors, such as the levels of protein acceptors, the levels of GPIT subunits, the levels of endogenous GPI phospholipase activity, and the levels of protease activity. In addition to these factors, solid tumors in different organs may sequester GPI anchored proteins into highly hydrophobic lipid raft membrane domains that are resistant to enzyme release. Based on our analysis of breast cancer tissue and plasma, increased levels of GPI anchored proteins were present in plasma from these patients. Breast cancers have frequent amplifications of chromosomal regions that contain the GPIT subunits. In Table 1, we list the subunits of the

GPIT and the chromosomal location of each. In addition, we indicate human cancers that have chromosomal amplifications in the regions that contain GPIT subunits [9–19]. Furthermore, we cite existing published data indicating increased expression for GPIT subunits in certain human cancers [1–3, 20–22]. The information in Table 1 indicates that the GPI transamidase is elevated in many human cancers, therefore we hypothesize that increased levels of GPI anchored proteins are present in plasma from patients with these malignancies. To test this hypothesis, we have analyzed plasma collected from breast, ovarian, kidney, liver, lung, colon, and brain cancer patients by performing slot blot followed by alpha toxin detection (Table 2, Fig. 3). An example, shown in Fig. 3A, indicates that GPI anchored proteins could be detected in plasma from breast, ovarian, kidney, liver, lung, colon, and brain cancer with no detection in plasma from patients without malignant disease (Table 2, Fig. 3). Each slot contained equivalent levels of plasma proteins as evidenced by the alpha-1 acid glycoprotein levels (Fig. 3A, right). We analyzed 10 samples from each cancer type except ovarian (6 cases) along with 12 plasma samples from patients without malignant disease. The non-malignant samples were composed of age-matched female controls to the breast cancer cases analyzed (10) and 2 samples from presumed healthy individuals no sex and age information available. Densitometry analysis of slot blots was performed, and the cumulative averaged alpha toxin signal intensities normalized to alpha-1 acid glycoprotein levels with SEM for each cancer are shown in Fig. 3B with value ranges shown for each sample in Table 2. These results indicate that GPI anchored proteins with a GPI anchor glycan attached were detected in the plasma from cancer patients at higher levels compared with plasma from patients without malignant disease. Variability exists in the levels of GPI anchored proteins detected by alpha toxin in different cancers and between different patients within certain cancer types. The cancers that show the highest variability are colon and brain cancer. Despite the patient variability, cumulative data indicate that GPI anchored proteins were detected in plasma at increased levels for all cancers analyzed (Fig. 3B). Therefore, proteomic studies to identify these GPI anchored proteins could lead to the discovery of novel biomarkers for cancer detection.

4. Discussion

GPI anchored proteins are highly conserved and vital for viability [23–25]. However, the levels of GPI anchored proteins in normal cells are under tight control evidenced by the lower levels of GPIT mRNA and protein levels in normal tissues and cells [4]. GPIT levels are amplified in human cancers due to chromosomal amplifications acquired during malignant transformation. The impact of how the amplification of GPIT subunits can influence cancer progression is just beginning to be assessed. Breast cancer studies indicate that elevations of GPIT lead to increased levels of GPI anchored proteins [4] and increased levels of tumorigenicity [1]. Based on these findings, we sought to determine whether the levels of GPI anchored proteins in plasma for various cancers correlates with previously described amplifications of GPIT levels in tissues for these cancers. Our results demonstrate that cancers with amplifications of certain GPIT subunits also have elevated alpha toxin binding to plasma from patients with these cancer types. We were surprised to discover that cancers with high GPIT mRNA and protein expression, such as breast and ovarian, do not have the highest levels of alpha toxin binding. For example, ovarian cancer has been shown

to have the highest levels of expression for GPIT subunits, catalytic and non-catalytic [3]. Yet, our data reveals that while GPI anchored proteins are detected by alpha toxin at higher levels in ovarian cancer compared with control plasma, the levels are lower than other cancers. These results illustrate that other factors contribute to release of GPI anchored proteins into plasma in a form that can be detected by alpha toxin. These factors may include possible amplification of protease activity or release by an endogenous GPI phospholipase that may result in a modified GPI anchor glycan region that is not recognized as avidly by alpha toxin. We have obtained proteomic data that indicate high levels of GPI anchored protein expression detected by alpha toxin binding to membrane proteins extracted from ovarian cancer tumors (data not shown) and the surface of cells isolated from patient ascites [26]; therefore, future studies to detail the differences in the GPI anchor structures of these proteins from tissue, ascites, and plasma may offer insight into why alpha toxin binding is lower for ovarian cancer plasma.

The functional significance of why GPI anchored proteins are released from the cell is not well understood. Studies from unicellular eukaryotic species have offered some insights into possible roles for released GPI anchored proteins [27]. Possible explanations include: greater cell to cell communication, a method to control antigenic variability, and control of cell shape influencing growth and migration characteristics of cells. Therefore, it is not difficult to envision how tumor cells would gain a survival advantage by releasing GPI anchored proteins. We have discovered that cancers from the colon and brain have the highest levels of GPI anchored proteins detected in plasma. Plasma obtained from patients with glioblastoma brain tumors and lower grade colon adenocarcinomas show the highest levels of alpha toxin binding. Increased release of GPI anchored proteins from these cancers may reflect higher levels of cell to cell communication such as synaptic activity in the brain, or a greater need to diversify cell surface antigens as observed in colonic epithelial cells.

In conclusion, our data documenting elevated levels of GPI anchored proteins in the plasma from human cancers indicate that this type of glycoconjugate is a potentially useful biomarker for the monitoring and detection of human cancers. We have demonstrated that alpha toxin can be used as a GPI-specific lectin to detect GPI anchored proteins in human plasma. Therefore, the identification of specific GPI anchored proteins for these cancers can foster the development of novel cancer detection methods and possible therapeutic strategies utilizing alpha toxin in the future.

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Fig. 1.

Diagram illustrating various mechanisms that can result in the release of GPI anchored proteins from the cell surface. GPI anchored proteins released from membrane-derived vesicles or via GPI-specific phospholipase enzymes would be expected to have an intact GPI anchor (lipid and glycan) or partial GPI anchor (glycan). GPI anchored proteins released by proteolysis will not have the GPI anchor.

Fig. 2.

Alpha toxin capture of CEA5 requires the presence of the GPI anchor. (A) LS174T human colon adenocarcinoma cells were divided into equal fractions, one fraction received buffer only (–) and one fraction received GPI-specific PI-PLC (1.5 units/ml) (+). Following incubation for one hour at 37°C, the supernatant was subjected to alpha toxin capture followed by Western blot detection of carcinoembryonic antigen 5 (CEA5). Input equals 10% of the supernatant used for alpha toxin capture. (B) Ten percent protein inputs and alpha toxin captured proteins were separated on a 4–12% polyacrylamide gel and stained with silver.

Fig. 3. Alpha toxin reacts with plasma from human cancers and not with control (non-malignant) plasma. (A) Representative slot blot analysis of human plasma (5 μ L) from patients with the indicated cancers detected with biotin labeled alpha toxin (2 μ g/ml) followed by streptavidin conjugated peroxidase and chemiluminescent substrate. (B) Cumulative results from slot blot analysis. Alpha toxin binding levels were normalized to alpha-1-acid glycoprotein levels for each case. Error bars indicate the $\pm$ SEM for normalized densities obtained from all plasma samples analyzed for each cancer (listed in Table 2).

Table 1

GPI transamidase subunits amplified in human cancers

GPIT subunit	Chromosome location	Cancers with amplifications of chromosome region	Cancers with demonstrated over-expression of GPIT subunit	References
PIGU	20q11.22	Bladder, breast, melanoma, ovarian, thyroid	Bladder, breast, colon, head and neck, kidney, lung, ovarian, skin, thyroid, uterus	Hyman E et al. 2002, Gorringe, kl et al. 2010, Guo Z et al., 2004, Brown et al., 2008, Yi-Jun Shen et al., 2007, Jiang et al., 2007, and Ishihara et al., 2008
PIGT	20q12-q13.12	Breast, esophageal, gastric, ovarian	Breast, colon, head and neck, ovarian, skin, thyroid, uterus	Fujita et al. 2003, Hidaka S et al. 2003, Sonoda G et al. 1997, Wu et al. 2006, Nagpal et al. 2008
GPAA1	8q24.3	Breast, colon, esophageal, hepatocellular, lung, prostate, uterine	Breast, bladder, head and neck, hepatocellular, kidney, lung, ovarian, prostate, uterine	Hyman E et al. 2002, Mark HF et al. 1999, Nagpal et al. 2008, Wu et al. 2006, Ho JC et al. 2006, Jiang et al. 2007
PIGK(GP18)	1p31.1	colon, hepatocellular	Bladder, breast, colon, liver, ovarian, prostate	Hashimoto et al. 2004, Daley D et al. 2008, Nagpal et al. 2008
PIGS	17p13.2	Gastric, multiple myeloma	Bladder, breast, lung, ovarian, uterine	Fabris S et al. 2007, Weiss MM et al. 2004, Nagpal et al. 2008

Table 2

Alpha toxin binding summary for plasma from patients with human cancers and controls

Cancer type	Histology	Grade	Number of cases	Alpha toxin binding value range
Breast	Invasive ductal	I	2	0.8–2.4
		II	5	0.8–2.4
		III	3	0.8–2.4
Ovarian	Serous	III	3	0.2–1.0
	Endometrioid	III	3	0.2–1.0
Kidney	Papillary adenocarcinoma	II	2	1.4–2.2
	Renal cell carcinoma	II	8	2.9–6.0
Liver	Adenocarcinoma	II	5	1.0–2.3
	Cholangiocarcinoma	III	1	2.3
	Carcinoid	unknown	3	3.5–7.0
	Sarcoma	unknown	1	1.9
Lung	Adenocarcinoma	I	1	2.2
		II	1	1.5
		unknown	2	2.1–2.5
	Squamous cell	II	3	3.1–6.0
	Non small cell	III	1	2
	Malignant mesothelioma	unknown	1	1.5
Colon	Adenocarcinoma	I	1	12
		II	8	2.1–4.0
	Adenocarcinoma in tubulovillous adenoma	II	1	9
Brain	Meningioma	II	3	4.1–6.1
	Glioblastoma	IV	2	8.1–9.0
	Oligosarcoma	unknown	1	3.2
	Gliosarcoma	IV	1	2.1
	Adenocarcinoma	unknown	1	2.1
	Glioma	II	1	1.2
Non-Malignant			12	0.1–0.2