THE ROLE OF ST6GAL-I SIALYLATION IN FAS (CD95) DEATH RECEPTOR FUNCTION AND TUMORIGENESIS.

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ABSTRACT

The golgi glycosyltransferase, ST6Gal-I, adds a negatively-charged sialic acid in an α2-6 linkage to N-linked glycans. ST6Gal-I is upregulated in many cancers, and is associated with increased metastasis and poor patient prognosis. ST6Gal-I expression has been shown to be driven by oncogenic-ras signaling. However, mechanistic details of the role ST6Gal-I plays in tumor initiation and progression are not well defined. Historically, studies have focused on contributions of ST6Gal-I to the malignant cell phenotypes of migration and invasion. Emerging evidence including studies contained in this dissertation have begun to elucidate a role for ST6Gal-I as a regulator of apoptotic signaling by providing protection against apoptotic stimuli. Additionally, we have found ST6Gal-I to be a potential regulator of stem cell populations in normal and cancer tissues.

Our work establishes the Fas receptor as a target for ST6Gal-I α2-6-linked sialylation and shows that this sialylation confers protection against Fas-mediated apoptosis. In both knockdown and forced expression cell models we demonstrated that ST6Gal-I protected cells from apoptosis as evidenced by decreased cleavage of caspases 3 and 8, and decreased morphological markers of apoptosis including nuclear condensation. Mechanistically, we found Fas sialylation blocked two vital steps of Fas-mediated apoptotic signaling, DISC formation and receptor internalization. Furthermore, we found ST6Gal-I upregulation resulted in hypersialylation of the Fas receptor in human colon tumors.
In addition to studies regarding functional outcomes of ST6Gal-I sialylation, we have begun to delineate ST6Gal-I protein expression and localization in several human epithelial tumors, including colon, prostate, ovarian, and pancreatic cancers. We have shown ST6Gal-I protein expression is upregulated in 14/15 colon carcinomas assayed, with highly upregulated staining in a majority of epithelial cells within immunostained tumors. ST6Gal-I expression appeared to localize to stem cell compartments within both normal colon and normal epidermal tissues and was also found to be upregulated in iPS cells. Additionally, cancer stem cell enrichment correlated with ST6Gal-I expression in two independent cell models.

This dissertation provides evidence regarding the role of ST6Gal-I in tumorigenesis and protection against apoptosis, and also raises the intriguing possibility that ST6Gal-I may contribute to tumor initiation through the regulation of stem cell phenotypes.

Keywords: sialylation, ST6Gal-I, Apoptosis, Glycosylation, Fas, stem cells
DEDICATION

I would like to dedicate this work to my best friend and husband, Grant. I could not have achieved this goal without your endless support and humor through many failed experiments and presentation rehearsals. Also, this is dedicated to my daughter, Natalie. Always pursue your dreams.
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## INTRODUCTION

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## SIALYLATION OF THE FAS DEATH RECEPTOR BY ST6GAL-I PROVIDES PROTECTION AGAINST FAS-MEDIATED APOPTOSIS IN COLON CARCINOMA CELL MODELS

1. ST6GAL-I PROTEIN EXPRESSION IS UPREGULATED IN HUMAN EPITHELIAL TUMORS AND CORRELATES WITH STEM CELL MARKERS IN NORMAL TISSUES AND COLON CANCER CELL LINES
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INTRODUCTION

N-linked Glycosylation

The surfaces of all eukaryotic cells are covered with a carbohydrate coating, the glycocalyx. This coating is composed of a complex variety of polysaccharide structures that have been secreted from the cell or covalently bound to proteins or lipids within the lipid bilayer. These structures are dynamically regulated in response to the cellular environment, and genetic mutations affecting glycan structure and composition can result in various disease states (1, 2). There are two main types of protein glycosylation in eukaryotes, N-linked and O-linked. N-linked glycosylation is the addition of a glycan to an asparagine (N) residue within a consensus sequence consisting of N-X-S/T, where X is any amino acid aside from proline. In O-linked glycosylation, the glycan is added at either serine or threonine residues (3). The focus of this dissertation is on N-linked glycosylation, and more specifically, the terminal α2-6-linked sialylation of the N-linked glycan.

N-linked glycans are added to proteins in the secretory pathway through post-translational modifications. These glycans are covalently attached to what will be the extracellular peptide sequence, and only consensus sequences that are exposed to the lumen of the endoplasmic reticulum (ER) are elaborated with N-linked glycans. One study has shown that approximately 60% of consensus sequences are occupied by a glycan, with occupancy being highly variable dependent on individual proteins (4). This high variability could be influenced by several factors including flanking amino acid
sequences, the identity of the X amino acid, whether the consensus sequence is NXS or NXT, and proximity to proline residues (4).

An immature glycan structure, consisting of a 7-sugar moiety, Man$_5$GlcNAc$_2$, is transferred as a block from a dolichol phosphate carrier embedded in the ER membrane to the asparagine residue of the target protein (Figure 1). This 7-sugar glycan is built on the dolichol phosphate acceptor on the cytoplasmic side of the ER and then “flipped” into the ER luminal space. Inside the lumen of the ER, the immature glycan will undergo modifications to form the 14-sugar structure Glc$_3$Man$_9$GlcNAc$_2$. This structure is then transferred to an asparagine residue of the nascent protein by the enzyme oligosaccharyltransferase (OST), which is the only enzyme known to add the 14-sugar moiety in N-linked glycosylation. After the glycan addition occurs, the sugar structure is further trimmed and modified as the glycoprotein continues to move through the ER and Golgi. In the Golgi, the glycan is tailored by various glycosidases and glycosyltransferases that can result in a wide array of mature glycans (5-8). Once these modifications are complete, it is not uncommon for an N-linked glycan to be multi-branched and can be several kiloDaltons in size (8).

The terminal galactose of these mature glycans can be capped with a variety of sugars including fucose, galactose, or sialic acid. These sugars are added in an alpha linkage, so they tend to project away from the beta-linked GlcNAc-Gal doublets that precede the capping sugar. This elaboration is conducted by a variety of enzymes expressed in the cis-, medial-, and trans- Golgi. A representative N-linked glycan is depicted in Figure 2. Due to the wide variety of possible linkages composing a mature glycan, the cell surface can be modified with a diverse glycan milieu, a phenomenon that
is termed microheterogeneity. Although the mechanisms that regulate the specific structure of these glycans are yet to be elucidated, we have nevertheless begun to characterize the effects of certain glycan modifications, with this dissertation focusing on the effects of sialic acid capping of the N-linked glycan in tumorigenesis.

Sialic Acids

Sialic acids are negatively charged 9-carbon sugar derivatives of neuraminic acid. There are approximately 50 different sialic acids, but the most commonly occurring in humans is N-acetylneuraminic acid (Neu5Ac), shown in Figure 3A (9). When capping N-linked glycans, the linkage can take place as an α2-3, α2-6, or α2-8 linkage (Figure 3B). α2-3 and α2-6 linkages are between a sialic acid and the terminal galactose, while α2-8 linkages occur when sialic acids are linked to other sialic acids, resulting in polysialic acid moieties (10, 11). α2-3- and α2-8-linked sialic acids are added by a variety of sialyltransferases, while ST6Gal-I was thought to be the sole enzyme responsible for the α2-6 linked sialic acid addition to N-linked glycans (12, 13). Recently, ST6Gal-II was identified as an additional enzyme capable of adding α2-6-linked sialic acids to N-linked glycans. However, up to this point ST6Gal-II expression has been shown to be much more restricted than ST6Gal-I with highest expression being in the brain, and the enzyme shows a preference for sialylating free oligosaccharides (14), which leaves ST6Gal-I as the major regulator of α2-6 sialylation of protein-bound N-linked glycans. Additionally, ST6Gal-II has not been demonstrated in human tumors, whereas ST6Gal-I mRNA expression has been shown to be highly upregulated in many malignancies, including colon carcinoma (14).
ST6Gal-I Sialyltransferase

ST6Gal-I, or β-Galactoside α2,6-sialyltransferase, is a type II transmembrane domain protein located in the trans-Golgi. ST6Gal-I has been shown to be regulated by the independent promoters P1-P4 with each resulting in a different expressed isoform varying in the 5’-untranslated regions. P1 (H isoform) is found in the liver and some colon cancer cell lines (15). P2 (X isoform) is expressed in mature B-lymphocytes (16). P3 (Y+Z isoform) is considered the housekeeping promoter, as this isoform is found at basal levels in multiple tissues (16). Of note, P3 (Y+Z isoform) is usually expressed in association with malignancy in a variety of cancers including colon cancer tissues and breast cancer cell lines (17, 18). The P4 is utilized exclusively in lactating mammary tissues and rarely is expressed in breast cancer cell lines (18).

Aside from transcriptional regulation, ST6Gal-I expression and α2-6 sialylation of N-linked glycans are also regulated by post-translational mechanisms. It has been assumed that α2-6 sialylation is directly correlated with ST6Gal-I expression. However, this is not completely accurate, as Dall’Olio et al. have shown that membrane α2-6 sialylation is not directly correlated with ST6Gal-I mRNA expression (17). This raised the possibility that α2-6 sialylation may be controlled by methods other than solely ST6Gal-I mRNA expression. Indeed, this regulation has been shown to take place through a variety of mechanisms. ST6Gal-I stability can be altered by oligomerization of the protein within the Golgi membrane (19). Also, work from our lab and others has shown ST6Gal-I protein to be cleaved by the BACE1 β secretase, presenting another method to control protein abundance (20-23). Along with ST6Gal-I expression, sialylation has been shown to be altered by a novel mechanism of α2-6 specific cleavage
in the extracellular space. A study by Cha et al. demonstrated that α2-6 sialic acids are cleaved from the TRPV5 Ca+ channels by the α2-6-linked sialic acid specific sialidase Klotho, which in turn reduces the channel’s stability on the cell surface (24). This study presents evidence that receptor-specific sialylation can be dynamically regulated in the extracellular domain whereas it was previously presumed that sialylation was a stable modification lasting the duration of glycoprotein surface expression.

**ST6Gal-I and Cell Differentiation**

ST6Gal-I expression levels vary in normal tissue and high levels have been correlated with an undifferentiated cell status. Several studies have demonstrated dynamic regulation of ST6Gal-I in immune cells upon differentiation. We and others have shown ST6Gal-I to be highly expressed in immature dendritic, monocytic, and T cells, whereas the expression is downregulated upon maturation or activation of these cells (23, 25-30). Outside of the immune system, this dissertation presents evidence of ST6Gal-I expression in non-differentiated cells, as we show high expression in induced pluripotent stem (iPS) cells. This is in concordance with a study by Tateno et al. demonstrating ST6Gal-I mRNA is highly expressed in both embryonic and iPS cells and is downregulated upon differentiation of these cells (31). Furthermore, data presented within this dissertation provide evidence of ST6Gal-I expression at the base of normal colonic crypts and in the basal layer of normal epidermis, which are both known to contain non-differentiated stem/progenitor cells that then differentiate as the cells move towards the terminally differentiated exposed surface (32, 33). While the mechanistic details of how ST6Gal-I is regulating or contributing to the progenitor/stem cell
phenotype are yet to be determined, these data show a correlation between ST6Gal-I and a relatively immature or undifferentiated phenotype. Also, they strongly support the role of ST6Gal-I as a major player in tumorigenesis, as tumor cells are often less differentiated than their non-malignant counterparts.

**ST6Gal-I and Cancer**

In addition to being associated with a less differentiated phenotype, ST6Gal-I has been shown to be highly expressed in several malignancies. ST6Gal-I mRNA and α2-6 sialylation activity have been shown to be upregulated in several types of cancers including colon, breast, acute myeloid leukemia, cervical, and gastric cancers (34-42). Our lab and others have shown expression to be upregulated by oncogenic-ras signaling (43-45). Furthermore, high expression and increased activity have been correlated with increased metastatic potential and poor patient prognosis in clinical studies involving colon and breast carcinomas (38, 46, 47).

Historically, the focus of study concerning the role of ST6Gal-I has centered on *in vitro* modeling of metastatic phenotypes including migration and invasion. Several studies from our lab and others have demonstrated that ST6Gal-I is correlated with increased invasion and migration (48-51). Specifically, we have shown that forced expression of ST6Gal-I in colon carcinoma and ovarian cell lines lacking endogenous ST6Gal-I expression increased collagen-I and laminin binding, and also increased migration towards collagen-I mediated by the hypersialylation of the β1 integrin (49, 51). In a separate study, we demonstrated that knocking down ST6Gal-I expression in colon carcinoma cells decreased adhesion to collagen-I and attenuated invasion through
Matrigel (50). This is in concordance with a study by Lin et al. which found that forcing expression of ST6Gal-I in breast cancer cells increased migration while decreasing cell-cell adhesion, whereas transfection with antisense shRNA for ST6Gal-I enhanced cell-cell adhesion (52). *In vivo* studies further support a role for ST6Gal-I in metastasis. Work by both Harvey et al. and Bresalier et al. show that high levels of α2-6 sialylation lead to a more metastatic phenotype in mouse xenograft studies (53, 54).

Aside from metastatic phenotypes, ST6Gal-I and resulting α2-6 sialylation have been shown to contribute to other characteristics associated with malignancy. Varki’s group has shown that spontaneous breast tumors were less differentiated in wild type mice than tumors from an ST6Gal-I null mouse (55). Also, colon cancer cell lines xenografted into mice usually demonstrate a higher ST6Gal-I activity than the original cell line (56). This finding suggests that ST6Gal-I may confer a survival advantage in cells, especially in the *in vivo* environment. This contention is further supported by work from our laboratory showing that ST6Gal-I protects against several mechanisms of cell death.

**ST6Gal-I and Protection from Apoptosis**

Cell surface sialylation has historically been suggested to play a role in cellular apoptosis. A wide array of studies have shown generic surface sialic acid content of cells is lower when the cells are undergoing apoptosis (57-59). These studies do not directly examine the effects of α2-6 sialylation on apoptosis, but rather suggest sialic acids in general may play a role in the regulation of apoptosis. However, work from our laboratory has addressed the more specific question of the role of ST6Gal-I and α2-6-
linked sialylation in apoptotic signaling (60-62). In this dissertation, the sialylation of Fas has been shown to protect against Fas-mediated apoptosis induced by both activating antibodies and the endogenous ligand FasL. Additionally, we have shown TNFR1 to be a target for ST6Gal-I sialylation that attenuates TNFR1 apoptotic signaling (60). In our work with β1 integrin sialylation, we found that ST6Gal-I α2-6 sialylation blocks galectin-3-mediated apoptosis by interfering with its binding to the β1 integrin (62). These studies highlight yet another role of ST6Gal-I in contributing to the tumor phenotype, providing many mechanisms by which the cancer cell can evade cell death.

Cell Death

There are currently three canonical cell death pathways: necrosis, apoptosis, and autophagy (63, 64). Necrosis takes place when the cell swells and bursts in response to stress such as nutrient depletion or membrane rupture. This is accompanied by an inflammatory reaction. There has recently been a surge of information regarding a variant of necrosis called controlled necrosis or necroptosis in which suicide signaling results in necrosis. This signaling is generally thought to transpire through RIPK1 activation (64). Apoptosis, in contrast to necrosis, is a highly regulated form of cellular death in which the mechanisms are tightly controlled. This form of cell death does not usually induce an immune response, but is rather a clean and efficient way of cell clearing. Apoptosis is characterized by membrane blebbing, nuclear condensation, and DNA cleavage. The clearance of these apoptotic cells takes place by phagocytes. Autophagy is less well characterized, and is defined by the cell “eating” itself, with the appearance of vacuoles within the cell and lack of nuclear condensation. This does not
always lead to cell death, but can provide a method of cell death when apoptosis is blocked (64). The emphasis of our studies has been on the apoptotic pathway.

Apoptosis

Apoptotic signaling is classified into two categories: intrinsic or extrinsic. Intrinsic signaling is also known as the mitochondrial pathway of apoptosis, as this pathway relies upon the mitochondrial loss of membrane potential resulting in the release of cytochrome c to carry out apoptosis (63). This can occur through a variety of signaling events, most depending on the interactions between BCL-2 protein family members (65). BCL-2 proteins are a family of proteins that share BCL-2 homology domains and are divided into three classes based upon their structure and function (Table 1).

Table 1: BCL-2 Family Members

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<tr>
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<th>Anti-apoptotic</th>
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<tr>
<td>BAX</td>
<td>BCL-2</td>
<td>BID</td>
</tr>
<tr>
<td>BAK</td>
<td>BCL-xL</td>
<td>BAD</td>
</tr>
<tr>
<td>BOK</td>
<td>BCL-W</td>
<td>BIM</td>
</tr>
<tr>
<td></td>
<td>MCL-1</td>
<td>BMF</td>
</tr>
<tr>
<td></td>
<td>A1/BFL</td>
<td>HRK</td>
</tr>
<tr>
<td></td>
<td>BOO/DIVA</td>
<td>BLK</td>
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Pro-apoptotic BCL-2 family members include BAX, BAK, and BOK. BAX and BAK are activated and translocate to the outer mitochondrial membrane where they form oligomers (66, 67). These oligomers create holes in the mitochondrial membrane and
cause mitochondrial outer membrane permeabilization resulting in cytochrome c release. Although BOK overexpression has been shown to kill cells, the specific roles of this protein and its ability to function like BAX and BAK are yet to be determined (68). The second class of BCL-2 proteins is the anti-apoptotic class, which includes many different members such as BCL-2 and BCL-xL. These function similarly to the pro-apoptotic class in that they are activated and embed in the outer mitochondrial membrane. Upon activation, these anti-apoptotic proteins can block the interaction between the pro-apoptotic BCL-2 class members, thereby prohibiting mitochondrial membrane permeabilization and apoptosis (69, 70). The third class of BCL-2 family members is the BH3-only class. These proteins contain the BCL-2 homology (BH) domain 3, which is homologous to the other BCL-2 family members and is also where their name is derived from. This class contains a variety of proteins that act as liaisons of a sort between the cellular environment and the pro-/anti-apoptotic BCL-2 classes to help initiate apoptosis (71). These proteins can be activated by cellular signaling events such as death receptor signaling and growth factor deprivation. Together, the interactions of these three classes of BLC-2 family members work to prevent or induce apoptosis by regulating mitochondrial membrane permeabilization and the release of cytochrome c (65).

Upon permeabilization of the outer mitochondrial membrane, the mitochondria releases cytochrome c which forms a complex known as the apoptosome. This complex includes cytochrome c, the adaptor protein APAF1, and the initiator caspase, caspase 9 (72). This complex formation leads to the cleavage of caspase 9, which goes on to cleave the executioner caspase, caspase 3 (73). Cleaved caspase 3 then goes on to cleave a multitude of substrates resulting in apoptosis for the cell (74). The regulation of this
process by BCL-2 family members is complex and has been well-reviewed elsewhere (65).

While the intrinsic pathway of apoptosis is heavily dependent upon the mitochondrial release of cytochrome c, the extrinsic pathway does not rely on the mitochondria but rather the activation of a sequence of caspases within the cytoplasm. The extrinsic pathway is initiated by extracellular signaling through what are commonly referred to as “death receptors.” These are transmembrane proteins classified in the tumor necrosis factor receptor (TNFR) superfamily. They include TNFR1, Fas, and Death Receptors (DR) 3-6 (75). The receptors are activated by endogenous ligands and then signal by the recruitment of a variety of adaptor proteins to their cytoplasmic tails. This recruitment leads to activation of caspases and apoptosis. To examine this type of apoptotic signaling in more detail, a member of this family, Fas (Apo-1, CD95) will be explained further as it is the focus of this dissertation.

Fas-Mediated Apoptosis

The Fas receptor is a member of the TNFR superfamily, and is the most widely characterized member of the death receptor family (76). Fas is a type I transmembrane protein, with its C-terminus in the cytoplasm and N-terminus in the extracellular domain. The extracellular portion of the receptor contains three cysteine-rich domains (CRDs) responsible for the extracellular interactions such as ligand binding (Figure 4). The endogenous ligand FasL binds to the receptor through CRD 2-3 while CDR 1 is thought to be responsible for the ligand-independent homotrimeric association of the receptor on the surface (77-79). This association occurs through what has been termed the preligand
binding assembly domain or PLAD, and is common for receptors in the TNFR superfamily. Upon ligand binding, the cytoplasmic tails of the receptor undergo a tertiary structure change (80). This allows for the recruitment and formation of the death-inducing signaling complex (DISC), which is composed of various proteins that bind to each other through homotypic interaction domains. The DISC provides the platform for downstream cytoplasmic signaling cascades.

Fas cytoplasmic tails contain a protein interaction region known as the death domain (DD) that binds to the DD of the adaptor protein, FADD (Fas-associated death domain). FADD has an additional protein-protein interaction domain, the death-effector domain (DED) which binds to other DED-containing proteins including caspase 8, caspase 10, and c-FLIP (81-85). Caspase 8 and caspase 10 are initiator caspases, meaning these proteases will cleave other downstream caspases known as executioner caspases (caspases 3 and 7). However, caspase 10 has been shown to exist in a 1:10 ratio compared to caspase 8 at the DISC, and is unable to initiate apoptosis in the absence of caspase 8 (86). Therefore it is thought that caspase 8 is likely the driver of apoptotic signaling at the level of the DISC. This formation of the DISC at the Fas cytoplasmic tails facilitates the homodimerization of caspase 8, leading to its cleavage and activation. The DISC formation capacity and amount of caspase 8 cleavage determines whether the intrinsic or extrinsic pathway of apoptosis will result.

Type I and Type II Cells

The Fas-mediated apoptotic response in a cell determines into which category the cell will be classified, either Type I or Type II cells (Figure 5). Type I cells follow the
extrinsic pathway of apoptosis and undergo rapid DISC formation. This results in high levels of caspase 8 cleavage, which are sufficient to cleave caspase 3 (87). The cleavage and activation of caspase 3 will then lead to apoptosis.

In Type II cells, the DISC is not as readily formed and therefore the level of caspase 8 cleavage is insufficient to cleave caspase 3. This type of cell will undergo the intrinsic pathway of apoptosis. Instead of cleaving caspase 3, caspase 8 targets the BH3-only protein BID for cleavage resulting in truncated BID (tBID). tBID will then cause BAX and BAD aggregation on the outer mitochondrial membrane and propagation of the mitochondrial pathway of apoptosis previously discussed (87). Both of these signaling cascades result in apoptosis despite the different methods used to reach the final endpoint. The cell lines discussed in the first part of this dissertation were found to be Type I cells based on lack of cleavage of BID and caspase-9 (data not shown).

Fas Receptor and Lipid Raft Localization

The Fas receptor has been shown to be present in lipid rafts in the cell membrane in both Type I and Type II cells (88). Lipid rafts are areas of the plasma membrane that are enriched for sphingolipids and cholesterol (89). Fas preferentially localizes to lipid rafts in Type I cells. However, in Type II cells Fas shows no preference for lipid raft or non-raft areas of the plasma membrane (88). Several studies have demonstrated Fas localization to lipid rafts to confer increased sensitivity to Fas-mediated apoptosis (90-92). Additionally, Fas-mediated apoptosis can occur in cells independent of ligand (FasL) engagement when Fas receptors are redistributed to lipid rafts (92, 93). Interestingly, the ether lipid edelfosine (a synthetic alkyl-lyso phospholipid anticancer
drug) induced apoptosis by causing the redistribution of Fas into lipid rafts and inducing ligand-independent Fas-mediated apoptosis (93). This has been shown to occur through the inhibition of phosphatidylinositol-3-kinase (PI3K) (94). Many studies indicate that disruption of the lipid rafts by cholesterol-depleting reagents can attenuate Fas-mediated apoptotic signaling (95-98). The role of lipid rafts in Fas apoptotic signaling has been questioned as a study by Algeciras-Schimnich et al. found that cholesterol-depleting reagents did not inhibit Fas-mediated apoptosis in B and T cells (99). However, a later study has shown that the aggregation of Fas receptors in lipid rafts after ligand binding and subsequent internalization of these clustered receptors resulted in dramatically increased caspase 8 cleavage (88). This study suggests that the lipid raft aggregation of Fas may play a role in efficient internalization of the Fas receptor, which is necessary for Fas apoptotic signaling.

**Fas Receptor Internalization**

After activation, the Fas receptor is internalized through clathrin-mediated endocytosis (99). It has been found that this internalization of Fas and the early DISC complex is required for apoptotic signaling (100). Lee et al. have shown in a very elegant sequence of experiments that Fas internalization happens rapidly after activation (either by FasL or agonistic antibody) and that this internalization sequesters the activated Fas receptor into endosomal and lysosomal compartments. Although a small amount of DISC assembly takes place at the membrane after activation, it was found that the majority of DISC assembly occurs after this subcellular localization (100). While this may seem counter-intuitive due to the concept that receptor internalization may serve to
abrogate signaling from the receptor, several studies have shown endosomal localization is a method to regulate and enhance resulting downstream signaling (reviewed in (101)). Furthermore, the study by Lee et al. goes on to demonstrate that signaling by the membrane localized (not internalized) Fas receptor results in pro-survival signaling such as activation of NFκB and ERK and an increase in motility and invasiveness (100).

**c-FLIP Regulates Fas Signaling**

Along with the caspases 8 and 10, c-FLIP is recruited to the DISC and binds to FADD through homotypic interactions. c-FLIP has been shown to be a major regulator of Fas-mediated apoptosis by interfering with caspase 8 activation (84). The c-FLIP protein is highly homologous to caspase 8 in sequence but c-FLIP lacks the activating cysteine residue important for caspase 8 protease activity. There are three isoforms: c-FLIP<sub>L</sub>, c-FLIP<sub>S</sub>, and c-FLIP<sub>R</sub>. C-FLIP<sub>S</sub> and c-FLIP<sub>R</sub> function in an anti-apoptotic manner while c-FLIP<sub>L</sub> can have pro- and anti-apoptotic functions (82, 102, 103). All of the isoforms contain two DEDs that mediate binding to FADD during DISC formation. At the level of the DISC, caspase 8 and c-FLIP form heterodimers and caspase 8 becomes activated, cleaving c-FLIP. However, c-FLIP cannot cleave caspase 8 so this interaction and cleavage of c-FLIP does not lead to apoptosis as the caspase 8 homodimerization can (84). Additionally, there are two cleavage products of the caspase 8 and c-FLIP interaction: p43-FLIP which results from caspase 8 cleavage of c-FLIP<sub>L</sub> at the DISC, and p22-FLIP which results from a death receptor-independent interaction of caspase 8 and c-FLIP in the cytoplasm. Both of these cleavage products activate NFκB activity by
binding to the IKK complex (104, 105), or by interaction with TRAF1, TRAF2 and RIP in the case of p43-FLIP (106).

Alternative Fas Signaling

The NFκB signaling downstream of the interaction between c-FLIP and caspase 8 highlights the complexity of Fas receptor signaling. While once considered only a death receptor in the most literal sense signaling only for apoptosis, there is paradigm shift occurring that demonstrates that Fas is capable of a variety of signaling cascades. Recent studies have shown that signaling through Fas can actually direct cell survival and proliferation dependent upon the cell or tissue type (107). For instance, activation of Fas signaling by the activating antibody Jo2 has been shown to be involved in the regeneration of liver after partial hepatectomy in mice (108). Along with liver regeneration, Fas signaling stimulated outgrowth of neurons by directing MAP kinase activation (109), and also induced dendritic and axonal branching in neurons of the central nervous system during early development (110).

In addition to the alternative functions of Fas in non-malignant cell types, these alternative signaling cascades are coming to the forefront in cancer as well. Fas is widely expressed by most types of cancers, yet many tumor cells are resistant to Fas-mediated apoptosis despite high levels of Fas receptor expression (111-116). This suggests there may be other roles for the Fas receptor in these cancer cells. In a landmark paper by Marcus Peter’s group, Fas signaling was shown to be vital for tumorigenesis and tumor survival (117). Additionally, another paper from this group has shown that Fas signaling can promote tumor progression by signaling through NFκB and the three major MAPK
pathways, ERK1/2, JNK1/2, and p38 (118). Along with proliferation and survival signaling, many studies have demonstrated that Fas signaling can also contribute to other tumor phenotypes such as migration and invasion (118-121).

The mechanisms by which the Fas receptor shifts from apoptosis to other signaling cascades are not fully understood. Interference with caspase cleavage by c-FLIP is one such mechanism. Also, TRIP6 has been shown to be recruited to the cytoplasmic tails of the Fas receptor thereby antagonizing DISC formation and promoting NFκB activity by direct interaction with NFκB p65 (120). Proliferation and survival signaling independent of the Fas-FADD interaction have also been identified in hepatocytes and thymocytes (108, 122). More recently, a similar phenomenon has been reported in pancreatic cancer cell lines (123). This study demonstrated that Fas-induced ERK signaling was not only FADD-independent, but that Src was actually recruited to the DISC in order to facilitate the ERK/Src survival signaling (123). These studies present several mechanisms for the opposing effects of Fas signaling. However, there are most likely many other mechanisms that regulate the signaling fates of the Fas receptor. Presented in this dissertation is a potential mechanism to describe how the Fas receptor can switch from apoptotic signaling to non-apoptotic tumor promoting signaling cascades through ST6Gal-I sialylation of the Fas receptor.

Fas and N-linked Glycosylation

The Fas protein contains two consensus sequences for N-linked glycosylation, both of which have been shown to carry N-linked glycans, N118 and N136 (Figure 4). The receptor has not been shown to carry any O-linked glycosylation (124). In this study
by Shatnyeva et al., it was hypothesized that the N-linked glycan at N136 could be important for ligand interaction. However, the study further confirmed what we had previously found, which is that alterations in these glycans do not interfere with the activation of the Fas receptor by changing the binding capacity of the ligand or activating antibody (124). The function of the N-linked glycan at N118 was proposed to be involved in stabilization of DISC-DISC interaction after ligand activation of Fas downstream signaling. Interestingly, this study found little effect of altered N-linked glycosylation on DISC formation, whereas other studies have shown that variation in sialylation specifically rather than generic N-linked glycosylation may contribute to regulation of Fas signaling (124). In a study by Peter et al., both B and T cells treated with neuraminidase to remove all cell surface sialylation were shown to be more susceptible to Fas-mediated apoptosis (125). Also, similar results have been shown in cancer cells as Keppler et al. showed B lymphoma cells with endogenous hypersialylation to be less affected by treatment with FasL and activating anti-Fas antibodies (126). These studies laid the groundwork for the data presented in the first part of this dissertation. In addition to these previous reports, we have identified that specific α2-6 sialylation of the Fas receptor driven by the sole enzyme ST6Gal-I attenuates Fas-mediated apoptosis by inhibiting two imperative steps in this pathway, DISC formation and receptor internalization.

Stem Cells

In addition to a role in regulating cell death, it became increasingly more evident as the project progressed that ST6Gal-I may be contributing much more to tumorigenesis
than solely protecting against apoptosis. In this dissertation we have identified ST6Gal-I expression to be associated with the stem/progenitor cell niche of normal colonic crypts and normal skin epidermis, in iPS cells, and also in cancer stem cell enriched populations.

The term “stem cell” covers a variety of cell types ranging from embryonic cells derived from pre-implanted blastocysts to the cells responsible for the regeneration of the epidermal skin layer. Unifying characteristics of these cells are that they have a unique regenerative capacity even after long periods of quiescence and they can produce daughter cells that may differentiate into a variety of cell types depending on the type of stem cell. Stem cells are broken down into three classes: embryonic, induced pluripotent stem cells, and somatic (also known as adult stem cells).

Embryonic stem cells, in terms of the cells used in research, are undifferentiated cells derived from a preimplantation stage blastocyst (127). These cells are pluripotent, meaning they have an unlimited differentiation capacity and can therefore give rise to any cell from the three germ layers: endoderm, mesoderm, and ectoderm. Another form of pluripotent stem cells is the induced pluripotent stem (iPS) cells. These are a newly discovered phenomenon found in 2006, which allows for the reprogramming of adult differentiated cells such as fibroblasts back into stem cells. Takahashi and Yamanaka have shown that by expressing a group of four genes (OCT4, SOX2, c-MYC, KLF4) in these cells, the cells can then be dedifferentiated and regain a pluripotent phenotype (128). These cells are an active area of research with a high level of therapeutic promise, as one could potentially regenerate necessary “replacement” tissues or blood instead of relying on transplants or transfusions (129).
Somatic stem cells are more specialized than embryonic and iPS cells in that they are not pluripotent, but can differentiate into several cell types based on what part of the adult body they are derived from. These cells are usually found amongst more differentiated cells within the organ or tissue type, and are responsible for regeneration of that particular organ. For example, colonic stem cells are found at the base of normal colon crypts, and serve to repopulate the crypts as cells move up the crypt towards the lumen and become terminally differentiated epithelial cells (33).

The data presented in the second part of this dissertation demonstrate that ST6Gal-I expression is associated with stemness in several stem cell niches. While this is the first report to link ST6Gal-I protein expression to the stem cell niche, ST6Gal-I mRNA and Sambucus nigra agglutinin (SNA, a lectin specific for the α2-6-linked sialic acid added by ST6Gal-I) activity have been reported in both iPS cells and embryonic stem cells (31, 130). Additionally, ST6Gal-I mRNA was found to be elevated in embryonic stem cells and to correlate with an undifferentiated phenotype in iPS cells (31). This is further confirmed by work in this dissertation showing high levels of ST6Gal-I protein expression in iPS cells.

Cancer Stem Cells

In addition to stem cells within nonmalignant tissues, tumors are hypothesized to contain a subset of cells termed cancer stem cells (CSCs) or tumor-initiating cells. This subset of cells is thought to be more aggressive and apoptosis-resistant than the “bulk” of the tumor. These cells were originally identified in leukemia by John Dick’s group which demonstrated that a small population of leukemic stem cells were able to cause the
disease in NOD-SCID mice (131). More recently, this tumor-initiating subset of cells has been identified in several types of solid tumors including colon cancer (132, 133). Cancer stem cells have been shown to be more resistant to chemotherapeutics (134), which is in concordance with data presented in this dissertation showing an increased stem cell enrichment in a chemo-resistant colon cancer cell line. These cells are typically identified using a variety of stem cell markers that may vary by tissue type. Two markers commonly used in colon carcinoma are ALDH1 and CD133. ALDH1 is an enzyme that acts as a detoxifier by oxidizing aldehydes, activity which may contribute to the hardiness of stem cells by protecting against alkylating agents (135). CD133 is a transmembrane glycoprotein for which the function is not well classified. It was originally identified by an antibody (AC133) raised against CD34+ hematopoietic stem cells (136). Some controversy remains as a report has shown that both CD133 positive and negative cells can initiate tumors in mice (137). In order to more confidently identify these populations, a combination of two markers is used. In this dissertation we have used these markers in combination to show that ST6Gal-I expression correlates with stem cell enrichment.

Research Overview

The purpose of this present work was to further elucidate the role of ST6Gal-I in tumorigenesis. The first section of this dissertation identified the Fas receptor as a target for ST6Gal-I. By using two independent genetically manipulated cell lines, both a knockdown and a forced expression model, we found that Fas does receive α2-6 sialylation via ST6Gal-I activity. Both cell lines were subjected to SNA precipitation and
Western blotting to isolate sialylated Fas receptors. Levels correlated with ST6Gal-I expression in all cases, where knockdown resulted in less sialylated Fas, and forced expression led to high levels of sialylated Fas. Additionally, 2D electrophoresis analysis confirmed that ST6Gal-I knockdown eliminated an acidic, high molecular weight isoform of Fas, presumed to be the sialylated form of the receptor. We also found that this sialylation of the Fas receptor attenuated Fas signaling at many levels. Increased ST6Gal-I expression led to decreased caspase 8 and caspase 3 cleavage. However, ST6Gal-I expression did not affect DR4/DR5-mediated apoptosis initiated by the endogenous ligand for these receptors, TRAIL, suggesting the ST6Gal-I driven protection against apoptosis is specific for the Fas receptor. Mechanistically, we demonstrated that ST6Gal-I sialylation inhibited Fas-mediated apoptosis by inhibiting two fundamental steps in apoptotic signaling. Co-immunoprecipitation studies showed that ST6Gal-I interrupted DISC formation by decreasing the recruitment of FADD to Fas cytoplasmic tails after receptor engagement with the Fas-activating antibody CH11. Furthermore, immunofluorescent labeling of the Fas receptor revealed Fas internalization was inhibited after activation in cells with high ST6Gal-I expression. These data were the first to establish that alterations in a single sialyltransferase could attenuate Fas-mediated apoptosis.

In addition to the mechanistic focus of the first work in this dissertation, we also began to examine ST6Gal-I protein expression and localization in human colon tumors, while still considering the role it may play in tumorigenesis. These studies were imperative in order to have a better understanding of ST6Gal-I expression patterns within normal and malignant human tissues. While many studies have identified ST6Gal-I
mRNA and activity through SNA labeling in various tissues, a major void existed in regards to actual protein expression within human tissues. This was mostly due to the lack of a reliable commercial antibody for ST6Gal-I. However, in the second section of this dissertation, a commercially available antibody was validated, and used to delineate ST6Gal-I protein expression in several human epithelial normal and malignant tissues.

We found through immunoblotting that ST6Gal-I was upregulated in seven out of eight colon carcinomas as compared to the pair-matched uninvolved colon tissues. Additionally, we found the Fas receptor to be hypersialylated in tumors with upregulated ST6Gal-I expression. Immunohistochemical analysis yielded invaluable information as to the expression patterns of ST6Gal-I within malignant and normal tissues. We found ST6Gal-I to be highly upregulated in seven out of seven colon tumors, with ST6Gal-I expression in the majority of epithelial tumor cells within the tumor tissue. We also found ST6Gal-I upregulation in several other epithelial tumors including pancreas, prostate, and stomach cancers. Interestingly, ST6Gal-I expression was confined to a very restricted number of cells at the base of the uninvolved colonic crypt, which also correlated with the known colon stem cell marker ALDH1. ST6Gal-I expression was also found to be highly upregulated in iPS cells as compared to the fibroblasts from which the iPS cells were derived. Furthermore, we demonstrated that knockdown of ST6Gal-I expression decreased cancer stem cell enrichment as identified by ALDH1+/CD133+ double-labeling. This would suggest a causal relationship between ST6Gal-I expression and the enrichment of cancer stem cells within a tumor cell population. Also, tying back to protection against apoptosis demonstrated in the first paper in this dissertation, we found that cells selected for chemoresistance had a drastic
increase in ST6Gal-I expression. These cells also displayed increased stem cell enrichment by double-labeling experiments, suggesting ST6Gal-I may confer a survival advantage within this population.

Together work in this dissertation sought to delineate mechanisms by which ST6Gal-I contributed to tumorigenesis, and to identify expression patterns within human cancers. We have shown that ST6Gal-I provides protection against Fas-mediated apoptosis, which is important in tumor cell survival. Furthermore, we have shown that ST6Gal-I protein is upregulated in human epithelial tumors, and functions to hypersialylate targets within those tumors. We have also demonstrated that ST6Gal-I may serve in a completely unexpected role in regulating stem cell phenotypes in normal and cancer stem cells. Collectively, data presented in this dissertation further define the role ST6Gal-I serves in tumorigenesis by showing it serves to regulate tumor cell survival and contributes to stemness in normal and cancer cells populations.
Figure 1: The Synthesis and Addition of N-linked Glycan to Asparagine.
1. Glycan synthesis begins on cytoplasmic side of the endoplasmic reticulum with the synthesis of the 7-sugar glycan core on a dolichol-phosphate molecule embedded in the ER membrane.
2. The 7-sugar core glycan is then flipped into the ER lumen where it undergoes further modifications that result in the 14-sugar moiety to be transferred to the glycoprotein.
3. The 14-sugar moiety is transferred to the newly synthesized protein within the ER lumen by oligosaccharyltransferase (OST).
Figure 2: Model of N-linked Glycosylation
1. The N-linked glycan is attached to the extracellular portion of a transmembrane protein.
2. Detailed depiction of an N-linked glycan. The glycan can be multibranched and the GlcNAc-Galactose pair can be repeated. The resulting glycan can be several kiloDaltons in size. The terminal galactose can be capped with a variety of sugars, one of which is the negatively charged sialic acid.
Figure 3: Sialic Acid Structure and Linkages

1. Most common sialic acid occurring in humans, N-acetylneuraminic acid (Neu5Ac).
2. Sialic acid linkages on an N-linked glycan can occur in α2-3, α2-6 or α2-8 bonds. α2-3 and α2-8 bonds are initiated by several sialyltransferases. ST6Gal-I is the primary sialyltransferase responsible for the α2-6 linkage on N-linked glycans.
The Fas receptor is a type I transmembrane domain protein. The extracellular portion of the protein contains three cysteine-rich domains or CRDs. These domains are responsible for interactions with FasL and activating antibodies. The cytoplasmic domain contains a death domain (DD), which binds to FADD through homotypic interactions with the DD within the FADD protein. This interaction serves as the platform for DISC formation and signaling cascades. N-linked glycosylation sites are indicated with blue arrows.
Figure 5: The Fas Apoptosis Signaling Cascades
Fas signaling can be classified as Type I, which follows the extrinsic pathway to apoptosis, or Type II, which carries out apoptosis through mitochondrial release of cytochrome c.
SIALYLATION OF THE FAS DEATH RECEPTOR BY ST6GAL-I PROVIDES PROTECTION AGAINST FAS-MEDIATED APOPTOSIS IN COLON CARCINOMA CELL MODELS.

by

AMANDA F. SWINDALL AND SUSAN L. BELLIS

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Format adapted and errata corrected for dissertation
Abstract

The glycosyltransferase, ST6Gal-I, adds sialic acid in an α2-6 linkage to the N-glycans of membrane and secreted glycoproteins. Up-regulation of ST6Gal-I occurs in many cancers including colon carcinoma, and correlates with metastasis and poor patient prognosis. However, mechanisms by which ST6Gal-I facilitates tumor progression remain poorly understood due to limited knowledge of enzyme substrates. Herein we identify the death receptor, Fas (CD95), as an ST6Gal-I substrate, and show α2-6 sialylation of Fas confers protection against Fas-mediated apoptosis. Intriguingly, differences in ST6Gal-I expression do not affect the function of DR4 or DR5 death receptors upon treatment with TRAIL, implicating a selective effect of ST6Gal-I on the Fas receptor. Using both ST6Gal-I knockdown and forced overexpression colon carcinoma cell models, we find α2-6 sialylation of Fas prevents apoptosis stimulated by FasL as well as the Fas-activating antibody, CH11, as evidenced by decreased activation of caspases 8 and 3. We also show α2-6 sialylation does not alter the binding of CH11 but rather inhibits the capacity of Fas to induce apoptosis by blocking the association of FADD with Fas cytoplasmic tails, an event that initiates death-inducing signaling complex formation. Furthermore, α2-6 sialylation of Fas inhibits Fas internalization, which is required for apoptotic signaling. Although dysregulated Fas activity is a well known mechanism through which tumors evade apoptosis, the current study is the first to link Fas insensitivity to the actions of a specific sialyltransferase. This finding establishes a new paradigm by which death receptor function is impaired for the self-protection of tumors against apoptosis.
Introduction

The ability to evade apoptosis is one of the defining characteristics of a malignant tumor cell (1). Escape from cell death can be accomplished through alterations in various cellular components including dysregulation of oncogenes and tumor suppressors, and mutations in apoptotic and anti-apoptotic signaling machinery. The TNF family of death receptors (TNFRs), including TNFR1, DR4, DR5, and Fas (CD95), represents one category of signaling molecules that is commonly disrupted in human tumors, and has been strongly implicated in tumor cell survival (2,3).

The Fas death receptor, like other TNFRs, is a homotrimeric transmembrane receptor that activates multiple intracellular signaling cascades, one of which directs apoptosis. Upon association with activating ligands, Fas undergoes higher order clustering, which facilitates the binding of cytosolic proteins to the Fas cytoplasmic tails. The first protein recruited to the Fas tails is FADD, which binds to Fas through a region known as the death domain. Several other proteins, including procaspase 8 and procaspase 10, are then recruited to the Fas/FADD complex, and together these proteins form the death inducing signaling complex (DISC). This complex is internalized through clathrin-mediated endocytosis, and allows for further DISC formation required for apoptotic signaling (4). This enhanced formation of the DISC leads to the autolytic cleavage and activation of procaspase 8, which goes on to cleave the effector caspase, caspase 3, ultimately resulting in apoptotic endpoints such as membrane blebbing and DNA fragmentation (2).

Diminished Fas expression and activity are well established as mechanisms responsible for the apoptotic resistance of tumor cells. A myriad of studies have reported
alterations in the expression of pro- and anti-apoptotic components involved in the Fas pathway such as c-FLIP, BAX, and BCL-2 (5-7). Additionally, in many tumor types there is up-regulation of the endogenous ligand for the Fas receptor, FasL, which is thought to provide a mechanism of self-conservation for tumor cells through FasL-directed killing of tumor-invading immune cells (8). However, despite the extensive research focused on changes in the expression level of Fas and associated signaling molecules, very few studies have investigated molecular mechanisms that alter Fas function independent of variant protein expression, for example, post-translational modifications such as glycosylation.

The Fas receptor is modified with both O- and N-linked glycans (9), although the functional significance of these glyconjugates in Fas signaling has received minimal attention. It has been known for decades that tumor cells express an altered profile of cell surface oligosaccharides, and in fact there is a specific subset of glycosylating enzymes exhibiting aberrant activity in human cancers. The ST6Gal-I sialyltransferase is one of the enzymes up-regulated in multiple types of cancer (10-15), and high ST6Gal-I levels are associated with increased metastatic potential (12,15-17). ST6Gal-I is responsible for the addition of the negatively charged sugar, sialic acid, in an α2-6 linkage to the N-linked glycans of cell surface or secreted glycoproteins. In the current study we have identified Fas as an ST6Gal-I substrate, and further determined that α2-6 sialylation of Fas inhibits Fas-mediated cell death. These collective results elucidate a novel mechanism by which tumor cells evade apoptosis.
Experimental Procedures

Cell Culture

HD3 colon epithelial cells expressing oncogenic ras were developed as previously reported (18). These HD3 cells were then transduced with lentivirus (purchased from Sigma) containing either shRNA sequence against ST6Gal (HD3.sh) or an empty vector (HD3.ev). A pooled population of clones stably expressing shRNA was generated by puromycin selection. Down-regulation of ST6Gal-I expression was confirmed by Western blot (19). SW48 cells were purchased from ATCC. These cells were transduced with either lentivirus containing rat liver ST6Gal-I cDNA (SW.ST6) or lentivirus containing an empty vector (SW.ev). The original ST6Gal-I plasmid was obtained from Dr. Karen Colley (University of Illinois, Chicago), and lentiviral vectors developed from this plasmid were constructed by Dr. John Wakefield (OpenBiosystems, Inc.). SW.ST6 cells represent a pooled population of stably-transduced clones, isolated by puromycin selection. Confirmation of expression and functionality of ST6Gal-I has been published previously (20). HD3 cells were maintained in low glucose (1g/L) DMEM with 7% FBS and 1% antibiotic/antifungal containing Penicillin G, Streptomycin sulfate, Amphotericin B (Gibco). SW48 cells were maintained in 1X-L15 medium with 10% FBS and 1% antibiotic/antifungal.

Western Blotting

To induce apoptosis, cells were treated with either 4ng/mL membrane bound FasL (Millipore), 0.5mg/mL CH11 (Millipore), or 2mg/mL TRAIL (Biomol International) for 18 h (Fig. s 1-3) or 6 h (Fig. 5C). Cells were then lysed in 50mM Tris-HCl (pH 7.4) with
1% Triton X-100 and protease inhibitors (Roche Applied Bioscience). Proteins were resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 5% dried non-fat milk in Tris buffered saline containing 0.01% Tween-20 (TBST) blocking buffer at room temperature for one h. The membranes were then subjected to primary antibody against: either cleaved caspase 3 (Cell Signaling Technology, Inc), cleaved caspase 8 (Cell Signaling Technology, Inc), Fas (Santa Cruz Biotechnology, Inc), FADD (Cell Signaling Technology, Inc), DR4 (Santa Cruz Biotechnology, Inc), DR5 (ProSci Inc), β-tubulin (abcam) overnight at 4°C. Membranes were washed and then subjected to HRP conjugated secondary antibodies diluted in blocking buffer for one h at room temperature. Proteins were visualized by Immobilon (Millipore).

Immunofluorescent staining

Cells were grown on chamber slides, and treated with the Fas-activating antibody, CH11 (Millipore) at 0.5mg/mL or TRAIL (Biomol International) at 2mg/mL for 18 h. Reaction was then stopped with ice-cold PBS. Protocol for Caspases 3&7 FLICA Apoptosis Detection Kit was followed as recommended by the manufacturer (Immunochemistry Technologies). The slides were imaged by bandpass filter with excitation at 550nm, emission at 580nm for the red fluorescence of cleaved caspases 3 and 7, and UV-filter with excitation at 365nm and emission at 480nm for Hoescht staining of the nuclei. For quantification, percentage of apoptosis was calculated as number of apoptotic cells compared to total number of cells in three independent fields of view. Statistical analysis was performed by repeated measure analysis of variance, with p<0.05 considered statistically significant.
**Lectin affinity analysis**

Cell lysate protein (1000µg) was incubated overnight with 50µL immobilized SNA-1 or MAA lectin (EY Laboratories) with rotation at 4°C. α2-6 sialylated proteins complexed with SNA or α2-3 sialylated proteins complexed with MAA lectin were collected by brief centrifugation, and washed. Sialylated proteins were released from the complexes by boiling in SDS-PAGE sample buffer. The proteins were then resolved by SDS-PAGE and immunoblotted to detect Fas.

**Two-dimensional Gel Electrophoresis**

HD3 whole cell lysates were prepared by lysing in lysis buffer with 50mM Tris-HCl (pH 7.4) with 1% Triton X-100 and protease inhibitors (Roche Applied Bioscience). Lysates were then diluted in IEF (isoelectric focusing) rehydration buffer containing 7M urea, 2M thiourea, 4% CHAPS, 30mM DTT, 0.5% ampholytes, and trace bromophenol blue. Samples were used to rehydrate 11cm 3-10 linear gradient IPG strips for 16 h in Drystrip Reswelling tray.

For the first dimension, IPG strips were focused using Amersham IPGphor II isoelectric focusing unit at 50µA per strip at 20°C. For the second dimension, strips were equilibrated in DTT equilibration buffer containing 6M urea, 20% glycerol, 50mM tris-HCl pH 8.8, 2% SDS, 65mM DTT and trace bromophenol blue for 30 min and in IA Equilibration buffer containing 6M urea, 20% glycerol, 50mM tris-HCl pH 8.8, 2% SDS, 2.5% iodoacetamide and trace bromophenol blue for 15 min. Strips were then electrophoresed on 12.5 % criterion gels (Bio-rad) at 100V constant in the Bio-rad
Criterion gel box. Proteins were then transferred to PVDF membranes and immunoblotted for Fas (Santa Cruz Biotechnology, Inc).

**Flow cytometry**

Cells were harvested nonenzymatically from tissue culture dishes using Cell Stripper solution (Cellgro). Cells were then resuspended into PBS containing 0.2% heat-denatured BSA. For antibody binding assay: cells were treated with CH11 (Millipore) at 20 µg/mL or IgM control for 1 h at 4 °C. Cells were then incubated with FITC-tagged secondary antibodies. For the CH11 binding curve, the same experiment was performed using a range of CH11 concentrations including 1, 10, 20 or 40 µg/mL. For Fas expression: cells were treated with FITC-conjugated anti-Human CD95 (BC Pharmingen) for 1 h at 4 °C. After labeling, cells were washed in PBS/BSA and then analyzed with FACSCalibur (Becton-Dickinson) by the UAB Arthritis and Musculoskeletal Center Analytic and Preparative Core Facility.

**DISC Immunoprecipitation**

Disc components were immunoprecipitated by treating 8.0x10^6 cells with 1.0 µg/mL CH11 (Millipore) in media at 4°C (control) or 37 °C for 30 min. Treatment was stopped by the addition of ice cold PBS. Cells were centrifuged to remove unbound antibody and washed with ice cold PBS. Cells were then subjected to lysis buffer containing 50mM Tris-HCl (pH 7.4) with 1% Triton X-100 and protease inhibitors (Roche Applied Bioscience) on ice for 15 min. Samples were centrifuged, and supernatant was collected and rotated overnight with 40µL prewashed anti-IgM conjugated agarose beads (Sigma).
Precipitated proteins were released from the complexes by boiling in SDS-PAGE sample buffer. Proteins were resolved by SDS-PAGE, and immunoblotted for the Fas-binding protein, FADD (Cell Signaling Technology, Inc). The PVDF membrane was then stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) and reprobed with Fas (Santa Cruz Biotechnology, Inc) as previously described.

**Internalization Assay**

Cells were lifted from the tissue culture plastic with trypsin, and trysin activity was stopped with trypsin inhibitor. Cells were resuspended in media with 1.0 µg/mL CH11 (Millipore) at 4°C (control) or were warmed to 37°C for 30 min to activate internalization. Cells were washed and resuspended in 3.7% formaldehyde in PBS and fixed for 15 min at room temperature. Cells were then washed and resuspended in media with 1.25µg/mL anti-mouse IgM Alexa 488 (Invitrogen) at 4°C for one h. Stained cells were then washed and resuspended in Vectashield (Vector Labs, Burlingame, CA), plated on a glass microscope slide. Slides were imaged with the Zeiss LSM 710 laser confocal scanning microscope.

**Results**

α2-6 sialylation confers protection against Fas-mediated apoptosis, but does not affect DR4/DR5 signaling.

To evaluate the role of ST6Gal-I-mediated sialylation in regulating tumor cell behavior, we previously developed a cell model system with variant levels of ST6Gal-I expression. HD3 colon carcinoma cells express high levels of endogenous ST6Gal-I as a secondary consequence of oncogenic ras activity (18). This cell line was subsequently
stably transduced with shRNA to force down-regulation of ST6Gal-I (19). In the current study, the parental HD3 cell line expressing high endogenous ST6Gal-I (HD3.par) and the shRNA knockdown of ST6Gal-I (HD3.sh) were treated with either the ligand for DR4 and DR5, TRAIL, or the Fas-activating antibody, CH11. Levels of apoptosis were then evaluated by immunoblotting for the apoptotic marker, cleaved caspase 3. As shown in Fig. 1A, when treated with CH11, HD3.sh cells exhibited a markedly greater degree of cleaved caspase 3 than HD3.par cells, suggesting that low levels of ST6Gal-I-mediated sialylation were associated with enhanced Fas-mediated apoptosis. In contrast, equally high levels of activated caspase 3 were observed in cells treated with TRAIL. Thus, variant levels of ST6Gal-I sialylation appeared to modulate apoptosis induced by Fas receptors, but not DR4 or DR5. To ensure that ST6Gal-I knockdown did not alter the expression of death receptors, Western blots of Fas, DR4 and DR5 were performed, which showed equivalent levels in the HD3.par and HD3.sh cells (Fig. 1B).

To further investigate the selective protection by ST6Gal-I against Fas-mediated apoptosis, we again treated the cells with TRAIL or CH11, and monitored apoptosis through immunofluorescence staining with the FLICA reagent, which binds to activated caspases 3 and 7 (cells were counterstained with Hoechst to reveal cell nuclei). HD3.par cells treated with CH11 displayed a small, insignificant, increase in FLICA staining relative to untreated HD3.par cells, however extensive FLICA staining was observed in the CH11-treated HD3.sh cells (Fig. 2A). In addition, CH11-treated HD3.sh cells exhibited condensed nuclei, and a disruption in the integrity of cell colonies, both indicative of apoptotic activity (Fig. 2B). In contrast to the effects of CH11, treatment with TRAIL induced very strong caspase activation in both the HD3.par and HD3.sh cells (Fig. 2C).
Analysis of percentage of apoptosis can be seen in Fig. 2D. These data suggest that sialylation by ST6Gal-I protects HD3 cells from apoptosis induced by the Fas receptor, but not DR4/DR5, and importantly, that ST6Gal-I knockdown can sensitize cells to Fas-mediated cell death.

α2-6 sialylation confers protection against ligand induced Fas-mediated apoptosis in two cell models.

Although the CH11 antibody is commonly used to stimulate Fas-mediated apoptosis, it was important to examine the effects of ST6Gal-I on apoptosis induced by the biologic ligand for the Fas receptor, FasL. HD3.par, HD3.sh, and additionally, an empty vector control cell line (HD3.ev), were treated with either CH11 or FasL, and immunoblotted for cleaved caspase 3. As shown in Fig. 3A, HD3.sh cells displayed higher levels of cleaved caspase 3 in response to both FasL and CH11 as compared with HD3.par and HD3.ev cells, confirming that diminished α2-6 sialylation renders cells more susceptible to two independent Fas activators.

Having shown that ST6Gal-I knockdown enhances Fas-mediated apoptosis, we next evaluated whether overexpression of the enzyme in cells with low endogenous ST6Gal-I would inhibit Fas-induced apoptosis. To this end we monitored cell death in the SW48 colon epithelial cell model, which lacks any detectable expression of ST6Gal-I (21). Previously we generated an SW48 cell line that stably expresses ST6Gal-I, and verified protein expression and enzyme activity (20). SW48 parental cells (SW.par), which have no ST6Gal-I, SW48 cells with forced expression of ST6Gal-I (SW.ST6), and an SW48 cell line transduced with an empty vector lentivirus (SW.ev), were treated with CH11 or FasL and surveyed for apoptosis (Fig. 3B). These experiments revealed sub-
stantially lower levels of activated caspase 3 in SW.ST6 cells compared with SW.par and SW.ev cells. Hence, the combined results in Fig. 3 establish that ST6Gal-I activity confers protection against apoptosis induced by both the Fas-activating antibody, CH11, and also the endogenous ligand, FasL, in two distinct colon carcinoma cell models.

*Fas receptor is a target for ST6Gal-I α2-6 sialylation.*

We next sought to determine if the Fas receptor is a direct target for α2-6 sialylation by ST6Gal-I, given that this receptor is known to have two possible N-linked glycosylation sites (22,23). To address this, cell lysates were incubated with agarose-conjugated SNA, a lectin that binds specifically to α2-6-linked sialic acids. Samples were centrifuged to selectively precipitate α2-6 sialylated proteins, and α2-6 sialylated proteins were then immunoblotted for the Fas receptor. As depicted in Fig. 4A, the band representing Fas in the SNA precipitates from HD3.par cells is denser than that noted in precipitates from HD3.sh cells, indicating more sialylated Fas in the presence of high ST6Gal-I expression. However, no differences were observed in the amount of Fas present in whole cell lysates (representing total Fas protein), indicating that variant ST6Gal-I expression alters Fas sialylation, but not Fas protein expression. Also, MAA lectin-precipitation (specific for α2-3 sialic acids) revealed that despite a decrease in α2-6 sialylation in the HD3 cells, there was no significant change in α2-3 sialylation of the Fas receptor (Fig. 4A). SNA precipitation analyses performed with SW48 cells yielded similar results; Fas was found to be heavily sialylated in SW48 cells with forced expression of ST6Gal-I (SW.ST6 cells), whereas the Fas receptor expressed by parental and empty-vector SW48 cells lacked α2-6 sialylation, as can be seen in Fig. 4B. (MAA
lectin-precipitation of SW48 cell lysate was not performed as these cells have no α2-3 sialyl transferases (21). As with HD3 cells, variant expression of ST6Gal-I did not alter total Fas protein levels.

To further confirm Fas as an ST6Gal-I substrate, whole cell lysates from HD3.par and HD3.sh cells were resolved using two-dimensional electrophoresis to separate proteins by both molecular weight and isoelectric point, and then immunoblotting was performed to detect Fas isoforms (Fig. 4C). Several Fas isoforms were revealed by this assay, however a higher molecular weight, more negatively charged band was missing from the HD3.sh lysates. These results are consistent with the loss of a sialylated Fas isoform in cells with ST6Gal-I knockdown. We also used flow cytometry to show that variant Fas sialylation did not change the levels of cell surface Fas expression (Fig. 4D).

α2-6 sialylation does not alter CH11 binding, but results in differences in DISC formation.

One possible explanation for the decrease in apoptotic signaling from sialylated Fas receptors was that sialic acids might sterically block the binding of Fas activators to the ligand-binding domain. We therefore examined the binding of CH11 antibody to the Fas receptor using flow cytometry. As shown in Fig. 5A, there was substantial, and equivalent, binding of CH11 to Fas expressed by the HD3.par and HD3.sh cell lines. To further investigate Fas-activator binding, we examined CH11 binding at a range of concentrations from 1-40 µg/mL. Both cell lines show comparable mean fluorescent intensity at every examined concentration (Fig. 5B). Thus, the strong inhibitory effect of sialylation on Fas-dependent apoptosis cannot be attributed to diminished binding of the
Fas-activating antibody. These data point to a role for sialylation in modulating some aspect of Fas receptor activation rather than ligand binding.

To examine the effect of ST6Gal-I sialylation on Fas signaling, HD3 cells were treated with CH11 and whole cell lysates were immunoblotted for cleaved caspase 8. Caspase 8, an initiator caspase, was evaluated because of its early recruitment to the DISC after Fas activation. As shown in Fig. 5C, the amount of cleaved caspase 8 is dramatically increased in CH11-treated HD3.sh cells as compared with the HD3.par cells, suggesting that Fas sialylation alters signaling at some step upstream of caspase 8 activation.

FADD is the initial protein recruited to the DISC, and binds directly to the cytoplasmic tail of the Fas receptor after activation. Therefore we examined the amount of FADD associated with the Fas receptor tails by co-immunoprecipitation experiments. HD3 cells were treated with CH11 at 37°C to activate DISC formation, and then the Fas receptor and the associated DISC complex were immunoprecipitated. As a control, cells were incubated with CH11 antibody at 4°C, a treatment that does not induce DISC formation. Immunoprecipitates were then blotted for associated FADD (Fig. 5D). These experiments showed that a basal amount of FADD was bound to the Fas cytoplasmic tails in control cells, and no apparent increase in Fas/FADD association was observed in the HD3.par cells upon activation by CH11. In marked contrast, CH11 treatment of HD3.sh cells at 37°C induced a substantial increase in FADD binding to the Fas cytoplasmic tails, indicative of DISC formation. These results suggest that sialylation of Fas somehow alters the accessibility of Fas cytoplasmic domains for binding to FADD, and consequently regulates the first step in DISC formation.
α2-6 sialylation limits Fas receptor internalization.

The internalization of Fas after receptor activation is necessary for Fas-mediated apoptotic signaling (4). Therefore, to further characterize the effects of receptor sialylation, we examined Fas internalization after treatment with CH11. We treated cells with CH11 at either 4°C (as a control) or 37°C (to allow for signaling and internalization), fixed the cells, and then used anti-mouse IgM Alexa-fluor 488 (Invitrogen) to visualize the Fas receptor remaining on the cell surface of non-permeablized cells. As can be seen in Fig. 6, no major differences were noted in surface Fas levels upon CH11 treatment at 4°C; however, upon receptor activation at 37°C, substantially more Fas was internalized in HD3.sh cells. These data suggest that α2-6 sialylation of Fas inhibits receptor internalization, thus limiting Fas-dependent apoptosis.

Discussion

There are multiple sialyltransferases that add sialic acid in an α2-3 linkage to N-glycans, however ST6Gal-I is the predominant enzyme that elaborates the α2-6 linkage of sialic acid to N-glycosylated proteins (24,25). Another α2-6 sialyltransferase, ST6Gal-II, has been identified, however this enzyme is localized primarily to the brain, and preferentially sialylates oligosaccharides rather than glycoproteins (26,27). There are also several ST6GalNAc enzymes that add α2-6 sialic acid to the GalNAc residue of O-linked glycans or gangliosides (reviewed in (28). The ST6Gal-I enzyme is overexpressed in at least 13 different types of cancers including colon, breast, esophageal, oral, ovarian, cervical, leukemias and brain tumors (10-15,29-31), and high ST6Gal-I levels are associated with metastasis and poor patient prognosis (15-17). The
functional contribution of ST6Gal-I to tumor progression has not been widely-investigated, however increased α2-6 sialylation has been linked to enhanced tumor cell migration and invasion (12,18-20,32). We and others have reported that ST6Gal-I expression is increased by oncogenic ras (reviewed in (33), which is found in over 30% of human cancers, and Piller’s group showed this up-regulation takes place through the ralGEF pathway (34). Studies of ST6Gal-I in colon carcinoma have revealed that 90% of colon tumors screened had up-regulated ST6Gal-I expression (11) and 70% of colorectal cancers were positive for the α2-6 sialic acid modification added by ST6Gal-I (35). Animal studies also support a role for ST6Gal-I up-regulation in tumor progression. Human (16) and murine (36) cancer cells with high levels of α2-6 sialylation were more metastatic to liver following splenic injection in nude mice, and enzymatic removal of sialylation from tumor cells prior to injection inhibited metastasis. Furthermore, Varki’s group reported that ST6Gal-I-null mice bred to a spontaneous breast cancer model displayed tumors that were more differentiated than tumors from wild-type mice, suggesting that ST6Gal-I activity contributes to the poorly-differentiated phenotype of more advanced cancers (37).

Despite these compelling results, there is still a limited understanding of the mechanism by which ST6Gal-I-directed sialylation regulates tumor cell behavior, because of the sparse knowledge of ST6Gal-I substrates, and more importantly, how the enzyme affects the function of specific proteins. The current investigation provides a significant advance toward defining the role of ST6Gal-I in tumor progression by showing that the Fas receptor is sialylated by ST6Gal-I. Furthermore, we show that α2-6 sialylation inhibits Fas signaling through both 1) blocking the binding of FADD to the
Fas cytoplasmic tail, which is the first step in DISC formation, and 2) inhibiting the internalization of the stimulated Fas receptor, which is necessary for Fas apoptotic signaling (4). Sialylation can alter receptor function in through several mechanisms, including conformational alteration (38), clustering (39), and differential internalization rate, depending on the specific receptor. Consistent with our work, the CD45 and PECAM receptors have been shown to be targets for α2-6 sialylation by ST6Gal-I, and this sialylation affects internalization in both cases (39-41).

The role of α2-6 sialylation in tumor progression has most often been associated with effects on tumor cell migration and invasion, however it is emerging that ST6Gal-I may be a major regulator of tumor cell survival. This sialylation-dependent survival benefit is likely mediated through multiple molecular pathways. Studies by our group and others have shown that ST6Gal-I-directed α2-6 sialylation of selected receptors serves as a key negative regulator of galectin-induced apoptosis (39,42,43). Additionally, the diminished internalization of PECAM due to α2-6 sialylation allows anti-apoptotic signaling form PECAM for a longer time interval (40). It has also been determined that ST6Gal-I levels are up-regulated after radiation treatment in mice (44), and these higher levels confer protection against radiation induced apoptosis. Our novel finding that α2-6 sialylation inhibits Fas signaling adds to the growing body of literature suggesting that ST6Gal-I modifies a select group of substrates to regulate a multiplicity of apoptotic signaling cascades, thus providing a strong selective advantage for tumor cells.

Changes in expression of the Fas receptor and associated signaling molecules have long been regarded as a mechanism by which tumors evade apoptosis. Several types of cancers are known to down-regulate the Fas receptor as a protective measure
including colon (45), testicular (46), and hepatoma (47). In addition, increased FasL expression on the tumor cell surface has been reported in several cancers (8), and is thought to play a role in tumor evasion from the immune system. Expression levels of downstream effectors in the Fas-induced apoptotic cascade are also altered in tumor tissue, providing additional mechanisms for protection against Fas-mediated apoptosis. Alterations include up-regulation of the anti-apoptotic Bcl and c-FLIP, and down-regulation of pro-apoptotic Bax (5-7). Interestingly, Fas is highly expressed on the surface of many tumor types that are not susceptible to Fas-mediated apoptosis (48-51).

Results presented herein implicate α2-6 sialylation as a newly identified mechanism by which tumor cells disable Fas signaling. A role for sialylation in regulating the Fas receptor was previously suggested by studies in lymphocytes (52,53). Keppler et al. reported that highly-sialylated subclones of the BJA-B lymphoma cell line were less susceptible to Fas mediated apoptosis, although α2-3, and α2-6 sialic acids were not distinguished in this study (52). Consistent with these results, Peter et al. determined that enzymatic removal of all cell surface sialic acids (i.e. both α2-3 and α2-6 sialylation) increased the vulnerability of B and T cells to Fas mediated apoptosis (53). While these prior studies clearly linked sialylation to Fas activity, they provided limited information regarding mechanism given that there are more than 20 different sialyltransferases. In the current study we show that Fas activity is regulated through α2-6 sialylation by a distinct sialyltransferase, ST6Gal-I, and of equal importance, this type of sialic acid modification has physiologic relevance given the known up-regulation of ST6Gal-I in cancer. Indeed we previously reported that another ST6Gal-I substrate, the β1 integrin, expresses elevated α2-6 sialylation in 100% of human colon tumors (20). It is also noteworthy that
the total levels of sialylation on the two cell model systems evaluated in this study are quite different: HD3 cells express α2-3 sialyotransferases (19), whereas SW48 cells express no endogenous sialyotransferases (21). Thus, ST6Gal-I-mediated sialylation of Fas blocks Fas signaling in cells with both extensive α2-3 cell surface sialylation, including α2-3 sialylation of the Fas receptor (HD3), and cells with no surface sialylation other than that directed by exogenously-expressed ST6Gal-I (SW48). These results imply a unique functionality imparted by the α2-6 sialic acid modification added by ST6Gal-I. Further highlighting the specificity of this novel molecular pathway, ST6Gal-I activity appears to have no effect on apoptotic signaling by the DR4 or DR5 death receptors. Intriguingly, work by Ashkenazi's group has shown O-glycosylation of the DR5 receptor regulates sensitivity to the DR5 ligand, TRAIL, but does not affect signaling by Fas (54). Moreover, the sites for O-glycosylation on DR5 are not conserved in the Fas receptor, and correspondingly, the DR5 receptor does not contain consensus sequences for N-glycosylation. A fundamental concept highlighted by these observations is that there is specificity in the effects of certain glycan structures on the function of distinct death receptors.

In conclusion, the current study is the first to demonstrate that a specific sialic acid modification, elaborated by an enzyme known to be up-regulated in cancer, inactivates signaling through the Fas receptor. These results have several important translational implications, including the potential for α2-6 sialylation to serve as a biomarker for Fas insensitivity. In addition, the finding that forced down-regulation of Fas α2-6 sialylation sensitizes tumor cells to apoptosis, even in cells expressing the powerful ras oncogene, suggests that ST6Gal-I may be a promising therapeutic target.
Finally, ST6Gal-I dependent regulation of Fas suggests a new paradigm in death receptor signaling, and it more broadly highlights an emerging role for ST6Gal-I as a critical mediator of multiple cell survival pathways.

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References


Figure 1: Apoptosis mediated by Fas, but not DR4/DR5 is altered by changes in ST6Gal-I expression.

A. After treatment with CH11 (Fas-activating antibody) or TRAIL (activating ligand for DR4/DR5), cell were lysed and lysates were subsequently Western blotted for cleaved caspase 3. The levels of cleaved caspase 3 are much higher in the HD3.sh CH11 treatment lane as compared to the HD3.par lane. There is little difference between the TRAIL treated HD3.sh and HD3.par samples. B. There is no change in total expression of DR4, DR5, or Fas. (Con: serum-containing control media, SF: serum-free media).
Figure 2: FLICA staining of cleaved caspases 3 and 7 demonstrates that ST6Gal-I protects cells from Fas but not DR4/DR5-mediated apoptosis. 
A. HD3.par and HD3.sh cells were stained with FLICA in the absence of treatment (panels 1 & 2). Upon treatment with CH11, there is more activated caspase 3 and 7 staining in HD3.sh (panel 4) as compared to HD3.par (panel 3). There are also changes in colony and individual cell morphology, indicating apoptosis. Additionally, as can be seen in B, there is nuclear condensation verifying apoptosis (white arrows) in the HD3.sh after treatment with CH11. C. To induce DR4/DR5 signaling, cells were treated with TRAIL. Minimal FLICA staining was observed in the absence of treatment (panels 1 and 2) whereas extensive and comparable amounts of cleaved caspases 3 and 7 were observed in the HD3.par (panel 3) and HD3.sh (panel 4) cells, indicating that ST6Gal-I activity does not protect against DR4/5-directed apoptosis (orange, FLICA staining of cleaved caspases 3 and 7; blue, Hoescht staining for nuclei). D. Percentage of apoptosis was quantified by counting FLICA-positive versus Hoescht-stained cells from multiple microscopic fields. *=p<0.01; **=p<0.001.
Figure 3: ST6Gal-I protects two distinct cell lines against CH11- and FasL-induced Fas-mediated apoptosis.

A. Knockdown of ST6Gal-I increases Fas-mediated apoptosis. HD3 cells were treated with either CH11 or FasL. Cell lysates were resolved by SDS-PAGE and immunoblotted for cleaved caspase 3. The levels of cleaved caspase 3 are higher in the HD3.sh cell lysate lanes upon treatment with CH11 and FasL. 

B. Forced expression of ST6Gal-I protects cells against Fas-mediated apoptosis. SW48 cells were treated with either CH11 or FasL. The lysates were resolved by SDS-PAGE and immunoblotted for cleaved caspase 3. Levels of cleaved caspase 3 are higher in the SW.par and SW.ev lanes upon Fas activation, indicating that the presence of ST6Gal-I protects cells from Fas-mediated apoptosis in both cell lines (HD3 and SW48).
Figure 4: Fas is a substrate for ST6Gal-I sialylation.  

A. Knockdown of ST6Gal-I reduces amount of Fas protein precipitated by α2-6, but not α2-3 linked, sialylation on Fas. HD3 cell lysates were incubated with either SNA lectin (specific for α2-6 sialic acids) or MAA lectin (specific for α2-3 sialic acids) each conjugated to agarose beads. Sialylated proteins were precipitated by centrifugation, and, after extensive washing, precipitates were resolved by SDS-PAGE, and immunoblotted for Fas. As shown, α2-6 sialylated Fas is present at a much higher level in the HD3.par as compared to the HD3.sh cells, whereas levels of α2-3 sialylation were very similar in the two cell lines. Whole cell lysates (i.e. not subjected to lectin precipitation) were also immunoblotted for Fas to reveal total levels of Fas protein.

B. Forced expression of ST6Gal-I induces α2-6 sialylation of Fas. SW48 cells were subjected to SNA precipitation as described in A. (MAA precipitation was not performed on SW48 due to the known lack of endogenous sialyltransferases in this cell line.) In A and B, the levels of α2-6 sialylated Fas present are correlated with ST6Gal-I expression, suggesting Fas to be a target for ST6Gal-I.
sialylation. C. Loss of a higher molecular weight, more acidic isoform in cells with ST6Gal-I knockdown. Whole cell lysates were subjected to resolution by two-dimensional SDS-PAGE. Proteins were transferred to PVDF membranes and immunoblotted for Fas. The more acidic, higher molecular weight isoform of Fas is not present upon the knockdown of ST6Gal-I (HD3.sh). D. Surface expression of Fas is not altered by α2-6 sialylation. Cells were stained with FITC-conjugated anti-Human Fas or FITC-conjugated isotype IgG control and characterized by flow cytometry. Surface expression of Fas is not altered as a consequence of differential ST6Gal-I mediated sialylation.
Figure 5: α2-6 sialylation of Fas inhibits DISC formation

A. Fas-activating antibody binds equally well to sialylated and unsialylated Fas receptor. HD3.par and HD3.sh cells were treated with CH11, and antibody binding was measured by flow cytometry. (Cells were treated at 4°C to prevent Fas activation.) The binding levels for both cell lines are very similar.

B. CH11 binding curve. Flow cytometric analysis of cells treated with CH11 at 1, 10, 20 or 40 μg/mL revealed comparable mean fluorescent intensities (MFI) at all concentrations. Values for MFI were normalized to the IgM control.

C. Caspase 8 cleavage is affected by variant Fas sialylation. Cells were treated with CH11, and lysates were resolved by SDS-PAGE, and immunoblotted for cleaved caspase 8. The level of cleaved caspase 8 is higher in the HD3.sh as compared with HD3.par upon treatment with CH11, indicating enhanced cleavage of caspase 8.
when ST6Gal-I is down-regulated. D. DISC formation enhanced by knockdown of ST6Gal-I. DISC components were isolated by treating cells at 4°C (control) or 37 °C for 30 min with CH11 and precipitating through anti-IgM conjugated agarose beads. Proteins were resolved by SDS-PAGE, and immunoblotted for the Fas-binding protein, FADD. As shown, there is more FADD in HD3.sh after Fas activation with CH11 at 37 °C, indicating more DISC formation in ST6Gal-I deficient cells. The PVDF membrane was reprobed for presence of Fas to verify equal immunoprecipitation. Densitometry was used to quantify the FADD and Fas bands, and the FADD:Fas ratio was calculated. Values shown represent the averages from two independent experiments.
Figure 6: α2-6 sialylation of Fas decreases Fas internalization. Cells were suspended and treated at 4°C (control) or 37 °C for 30 min with 1 µg/mL CH11. Cells were then fixed and secondary anti-mouse IgM Alexa-fluor 488 (Invitrogen) was used to visualize the remaining Fas receptors on the surface of non-permeabilized cells. As can be seen in the HD3.sh cells (bottom panels), there is extensive internalization of the Fas receptor at 37 °C, which is not seen in the HD3.par cells (top panels).
ST6GAL-I PROTEIN EXPRESSION IS UPREGULATED IN HUMAN EPITHELIAL TUMORS AND CORRELATES WITH STEM CELL MARKERS IN NORMAL TISSUES AND COLON CANCER CELL LINES

by

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Abstract

The ST6Gal-I sialyltransferase adds an α2-6-linked sialic acid to N-glycans of selected receptors. ST6Gal-I mRNA is reportedly upregulated in multiple human cancers, however, the prior lack of antibodies limited characterization of ST6Gal-I protein. Here we show upregulated ST6Gal-I protein in several epithelial cancers, including colon carcinoma. Immunoblotting analyses revealed that 7/8 human colon tumors had elevated ST6Gal-I, whereas 7/7 tumors evaluated by immunohistochemistry overexpressed ST6Gal-I. In normal colon, ST6Gal-I was selectively localized to the base of crypts, the known site for stem/progenitor cells, and the staining pattern was similar to that of the stem cell marker, ALDH1. Similarly, ST6Gal-I expression was restricted to the basal layer of epidermis, another stem/progenitor cell compartment. ST6Gal-I was highly expressed in induced pluripotent stem (iPS) cells, with no detectable expression in the fibroblasts from which iPS cells were derived. Considering this apparent stem cell localization, we investigated whether ST6Gal-I was associated with cancer stem cells (CSCs). A colon carcinoma cell line, SW948, selected for resistance to the chemotherapy drug, Irinotecan, demonstrated a greater proportion of CSCs compared with parental cells, measured by labeling of CSC markers, CD133 and ALDH1 activity (Aldefluor). Correspondingly, chemoresistant cells exhibited marked ST6Gal-I upregulation. We also evaluated HD3 colon carcinoma cells with high endogenous ST6Gal-I and found stable knockdown of ST6Gal-I by shRNA significantly decreased the number of CD133/ALDH1-positive cells. Collectively these data suggest a role for ST6Gal-I in tumorigenesis and point to ST6Gal-I as a possible regulator of the stem cell phenotype in both normal and cancer cell populations.
Introduction

Differences in the glycan profile of cancer cells as compared with normal cells are well documented. These changes are driven by various enzymes responsible for the addition and removal of sugars, such as glycosyltransferases and glycosidases. There is a selected subset of enzymes altered in cancer, suggesting a functional role for distinct glycans in the tumor phenotype. The ST6Gal-I sialyltransferase is an example of a glycosyltransferase commonly upregulated in cancer. This Golgi enzyme adds the negatively-charged sugar, sialic acid, in an α2-6 linkage to the termini of N-linked glycans. Previous studies have shown ST6Gal-I to be upregulated in many types of cancers including colon, breast, ovarian, and cervical carcinomas, and overexpression correlates with increased metastatic potential and poor patient prognosis (reviewed in (1-3)). ST6Gal-I is upregulated in cancer as a consequence of signaling by the ras oncogene (1-3).

The mechanistic role of ST6Gal-I in tumor progression remains poorly-understood despite the numerous reports of increased ST6Gal-I expression in cancer. In vitro studies suggest that ST6Gal-I promotes cell migration and invasion (4, 5), and this enhanced migratory response is due, at least in part, to ST6Gal-I-mediated sialylation of the β1 integrin receptor (6-8). Animal models also implicate ST6Gal-I in tumor invasiveness. Bresalier et al. determined that metastatic murine cell lines were more highly sialylated than the less metastatic parental lines, and neuraminidase treatment of the metastatic lines drastically decreased the amount of liver metastases after splenic injection (9). Also, Harvey et al. reported decreased metastasis to liver following splenic injections after blocking the transfer of sialic acid from its carrier, CMP-sialic acid (10).
In conjunction with cell migration, ST6Gal-I may regulate another important aspect of tumorigenicity, the ability to evade cell death. Work from our group revealed that the Fas death receptor is a substrate for ST6Gal-I, and that α2-6 sialylation of Fas reduces apoptotic signaling by hindering internalization of Fas after ligand-induced activation (11). We similarly reported that ST6Gal-I-mediated sialylation of the TNFR1 death receptor blocks TNFα-induced apoptosis (12). Baum’s group showed that sialylation of CD45 by ST6Gal-I prevents CD45 internalization, thereby protecting T-cells from apoptosis (13), and ST6Gal-I sialylation of the PECAM receptor enhances PECAM surface retention, promoting survival of endothelial cells (14). These studies highlight the capacity of ST6Gal-I to modulate the function of specific receptors, particularly through regulation of cell surface retention. However, additional evidence has established ST6Gal-I as a key negative regulator of galectin-dependent apoptosis. Galectins are galactose-binding lectins that have many functions, including induction of cell death. The addition of α2-6 sialylation to galactosides prevents galectin binding and apoptotic activity (15). For example, our studies have shown that galectin-3 binds directly to the β1 integrin and stimulates apoptosis, but only when the β1 integrin lacks α2-6 sialylation (16). Finally, sialylation of EGFR by ST6Gal-I confers resistance to the EGFR-targeted chemotherapy reagent, gefitinib (17). These diverse findings suggest that ST6Gal-I acts as a critical regulator of tumor cell survival by inhibiting a multiplicity of cell death pathways.

While studies of specific receptors and signaling pathways have provided insight into the function of ST6Gal-I within a cellular context, a major gap in our knowledge is that ST6Gal-I expression in normal and tumor tissues has not been well-characterized.
Due to a prior lack of effective anti-ST6Gal-I antibodies, the vast majority of studies utilized measurements of ST6Gal-I mRNA levels, or tissue reactivity toward SNA, a lectin specific for α2-6 linked sialic acid. However, there are limitations associated with both of these approaches. The mRNA pool isolated from tumor tissue homogenates may include mRNA from noncancerous cells such as immune or endothelial cells, and the SNA lectin binds to α2-6 sialic acids added by multiple enzymes including ST6Gal-I and ST6GalNAc family members. To address this issue, immunohistochemical and immunoblot analyses of ST6Gal-I protein were performed in the current study using a newly-validation antibody, which revealed a dramatic upregulation of ST6Gal-I in tumor specimens compared with pair-matched uninvolved tissues. Surprisingly, the expression of ST6Gal-I in normal epithelium appeared to be localized to the stem and/or progenitor cell compartment, and moreover, high ST6Gal-I levels corresponded with the expression of the cancer stem cell markers, CD133 and ALDH1. While many questions remain regarding ST6Gal-I function in cancer, these data hint that ST6Gal-I activity may be involved in maintaining some aspect of stem-like cell behavior.

Methods

Cell Cultures

HD3 colon carcinoma cells were developed as previously reported (18) and maintained in Dulbecco’s Modified Eagles medium (DMEM) low glucose (1 g/L) with 7% FBS and 1% antibiotic/antifungal containing streptomycin sulfate, penicillin G, and amphotericin B (Invitrogen, Grand Island, NY). The stable ST6Gal-I knockdown cell line was established as previously described (8). In brief, the cells were transduced with
lentivirus (Sigma, St. Louis, MO) which contained either the shRNA sequence against ST6Gal-I or an empty vector. The cells were selected by puromycin, and a pooled population of clones stably expressing shRNA was maintained in 0.5 mg/mL puromycin containing media. ST6Gal-I downregulation was confirmed by Western blot.

SW948 colon carcinoma cells were purchased from ATCC (Manassas, VA). Cells were maintained in DMEM: Liebovitz’ L-15 medium in a 3:1 ratio, with 10% denatured FBS and 2mM glutamine. In order to establish a chemoresistant cell subline, SW948 cells were treated with an initial dose of CPT-11 (Irinotecan hydrochloride, Pharmacia & Upjohn Co., Kalamazoo, MI) at 4 mg/mL, which is 2-fold the determined IC$_{50}$ dose. Most cells were killed at initial dosing by day 10. Surviving cells were allowed to grow in drug-free media for 3 days. CPT-11 (4 mg/mL) was added back to the media for 5 days. After a short recovery period in drug-free media (3 days), cells were capable of growth in CPT-11 containing media (4 mg/mL). Dosage was then increased stepwise for a period of 185 total days reaching a maximum of 20 mg/mL. Cells were then cultured in 3:1 DMEM:L15 media containing 20 mg/mL CPT-11. Cells were periodically examined for drug resistance. Cells maintained resistance to CPT-11 even after growth in drug-free media out to 122 days.

Sample preparation and ST6Gal-I immunoblots

Colon tumor blot: Commercially available membrane containing three human colon tumor samples, one normal colon and one placental control was purchased from Biochain Institute (Newark, CA). Tumor and pair-matched uninvolved colon specimens:
Human tissues were obtained from the UAB Tissue Procurement Facility at the University of Alabama at Birmingham. These samples were homogenized using a polytron device in 50 mM Tris-HCl buffer (pH 7.4) with 1% Triton X-100 and protease inhibitors (Roche Applied Bioscience, Indianapolis, IN). Samples were centrifuged and supernatants used for immunoblotting. *iPS or HFF cell lysates:* Frozen cell lysates were purchased from Systems Biotechnologies. *Colon carcinoma cell lines:* HD3 and SW948 cells were lysed in 50mM Tris-HCl buffer (pH 7.4) containing 1% Triton X-100 and protease inhibitors. Lysates were centrifuged and supernatants collected for immunoblotting.

For the various preparations described above, samples were separated by SDS-PAGE, and transferred to PVDF membranes. Membranes were blocked in 5% dried non-fat milk (NFM) in Tris buffered saline containing 0.01% Tween-20 (TBST) at room temperature for one hour. The membranes were then incubated overnight at 4°C with primary anti-ST6Gal-I antibody (catalogue # AF5924, R&D Systems, Minneapolis, MN), used at a concentration of 1 mg/mL and diluted into TBST containing 5% NFM. Membranes were washed in TBST and incubated with HRP conjugated secondary antibody (diluted in 5% NFM/TBST) for one hour at room temperature. Protein signal was developed with Immobilon (Millipore, Billerica, MA). To control for protein loading, membranes were stripped and re-blotted for GAPDH (Cell Signaling Technologies, Danvers, MA), or β-actin (Cell Signaling Technologies).
**SNA Precipitation and Fas immunoblots**

Human tissue samples (uninvolved and tumor tissue) were homogenized as described above. Protein homogenate was incubated overnight with 50 mL SNA-1 conjugated to agarose (EY Laboratories, San Mateo, CA) with rotation at 4°C. α2-6 sialylated proteins complexed with SNA lectin were collected by brief centrifugation, and washed. Sialylated proteins were released from the complexes by boiling in SDS-PAGE sample buffer. The proteins were then resolved by SDS-PAGE and immunoblotted for Fas (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). To evaluate levels of total Fas protein, Fas immunoblots were performed using aliquots of the initial tissue homogenates (not subjected to SNA precipitation). Densitometry analysis on mature Fas (upper bands) was conducted using Image J software.

**Immunohistochemistry**

Slides with paraffin embedded pair-matched tumor and uninvolved colon tissue were obtained from Biochain Institute (Newark, CA). Slides were rehydrated using xylene and a gradient of EtOH solutions including 100%, 95%, 80% and 70% EtOH in DiH₂O for 5 minutes each. Slides with frozen multi-tissue arrays were purchased from Biochain Institute (Newark, CA) and thawed at room temperature. Antigen retrieval was performed by boiling slides in citrate buffer (Vector Labs, Burlingame, CA) for 30 minutes. Slides were allowed to cool at room temperature for 60 minutes. Slides were blocked for 60 minutes in 10% normalized horse serum diluted in phosphate-buffered saline (PBS). The following antibodies were then applied overnight at 4°C: ST6Gal-I (R&D Systems) used at 5 mg/mL, or ALDH1 (BD Pharmingen, San Jose, CA) at 2.5
mg/mL, each diluted into blocking buffer. Slides were washed in PBS and secondary antibody was applied for thirty minutes at room temperature (Immpress, Vector Labs). Slides were then developed with Immpact NovaRed (Vector Labs), and counter-stained with hematoxylin (Vector Labs). Slides were dehydrated through 70%, 85%, 95% and 100% EtOH, and xylene. Slides were then fixed with Permount (Vector Labs), and imaged with a Nikon compound microscope. Images were captured with ISCapture software.

**Validation of ST6Gal-I antibody**

Specificity of the ST6Gal-I antibody (R&D Systems #AF5924) was validated by immunoblotting and immunofluorescent staining of two established cell lines with forced expression of ST6Gal-I (Fig. S1). SW48 colon cancer cells and OV4 ovarian cancer cells have been shown to have undetectable endogenous ST6Gal-I expression, and ST6Gal-I expression was forced in these lines as previously reported (6, 7). (Of note, SW48 and SW948 are distinct colon cancer cell lines.) Immunoblotting was conducted using conditions described above, and immunofluorescent staining was performed using 1 mg/mL anti-ST6Gal-I antibody, followed by Alexa-conjugated secondary antibody (Life Technologies, Grand Island, NY). Immunohistochemical staining was performed on both the OV4 cell line and human tissues (Fig. S2). Frozen slides containing colon metastasis to liver were subjected to antigen retrieval and immunostaining as described above. Samples were incubated with either primary antibody or an isotype control antibody. As an additional control, paraffin-embedded uninvolved colon and colon tumor specimens
were prepared as previously described and exposed to secondary antibody alone (no primary).

Flow Cytometry

Cells were detached from tissue culture flasks by brief trypsinization. \(1 \times 10^6\) cells were counted and treated for analysis of ALDH1 activity using the Aldefluor activity assay as recommended by the manufacturer (StemCell Technologies, Vancouver, BC). Samples from each cell line with inhibited Aldefluor staining were used as the gating control. CD133/1-PE antibody (AC133) was used to detect CD133 according to the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA). Results were gated for non-specific activity by isotype control (IgG1, Miltenyi Biotec). Cells were then analyzed by flow cytometry for stem cell markers with a FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ) at the University of Alabama at Birmingham Rheumatic Diseases Core Center (RDCC) Analytic and Prepartive Cytometry Facility (APCF).

Statistical Analysis

For flow cytometry analysis, the proportion of labeled cells as compared to the total number of cells within the population was compared using a z test for two proportions. \(p\) values less than 0.05 are considered significant.
Results

ST6Gal-I upregulation in human colon tumors

In order to address the lack of information regarding ST6Gal-I protein expression in human tissues, we screened several new commercial antibodies and identified one that reliably detects ST6Gal-I protein (Fig. S1 & S2). Using this antibody, we evaluated ST6Gal-I levels in human colon cancer tissues using a commercial membrane blot containing three independent cases of human colon carcinoma, along with normal colon and normal placenta controls. As shown in Figure 1A, higher ST6Gal-I expression was observed in the colon tumors as compared with normal colon and placental tissues. The upper band on the blots represents full-length ST6Gal-I, while the size of the lower band is consistent with the cleaved, secreted form of ST6Gal-I.

We next examined ST6Gal-I expression in tumor and pair-matched uninvolved colon specimens obtained from the Tissue Procurement Shared Facility of the Comprehensive Cancer Center at the University of Alabama at Birmingham. Tissues were homogenized and immunoblotted for ST6Gal-I. Four of the five patient samples exhibited upregulation of ST6Gal-I in the tumor compared with the cognate uninvolved specimens (Fig. 1B).

Elevated α2-6 sialylation of the Fas receptor in human colon carcinoma samples

To assess the functional consequence of ST6Gal-I upregulation in tumors, we measured the levels of α2-6 sialylation on the Fas receptor. Utilizing patient samples for which adequate tissue homogenate was available, tumor and pair-matched uninvolved colon tissue homogenates were incubated with agarose-conjugated SNA-1 lectin. The α2-
6- sialylated proteins bound by SNA-agarose were isolated by centrifugation, resolved by SDS-PAGE and immunoblotted for Fas (Fig. 2A, top panels). To measure total Fas expression, samples of the original tissue homogenates (not subjected to SNA precipitation) were immunoblotted for Fas (Fig. 2A, bottom panels). We found that total Fas expression was decreased in the tumors, consistent with other studies suggesting that Fas is downregulated in colon carcinoma as a mechanism for protection against Fas-mediated apoptosis (19). However, despite Fas downregulation, the proportion of α2-6 sialylated Fas in the tumor tissues was distinctively higher than the proportion of α2-6 sialylated Fas in uninvolved colon tissues for those cases that exhibited ST6Gal-I upregulation (patients 4 and 5, Fig. 2B). Conversely, levels of α2-6 sialylated Fas were comparable in tumor and uninvolved tissues from the patient sample that did not exhibit ST6Gal-I upregulation (patient 8). Thus, ST6Gal-I overexpression in tumors acts to hypersialylate Fas despite an overall downregulation in Fas protein. Hypersialylation of Fas, which inhibits Fas receptor internalization and apoptotic signaling (11), may constitute a second line of defense in tumors through blocking the activity of Fas receptors remaining on the tumor cell surface. The determination that Fas has enhanced α2-6 sialylation in tumors is consistent with our prior studies showing that β1 integrins exhibit elevated α2-6 sialylation in tumor tissues (6). These results confirm that upregulation of ST6Gal-I in tumors leads to elevated α2-6 sialylation of functionally-important ST6Gal-I targets.
**ST6Gal-I upregulation and localization in colon tumors**

Although ST6Gal-I upregulation in colon tumors was observed by immunoblotting, this does not address protein localization. To determine the tissue localization of ST6Gal-I, paraffin-embedded pair-matched tumor and uninvolved colon tissues from seven patients were stained by immunohistochemistry to visualize ST6Gal-I protein. Shown in Figure 3A are uninvolved colon and tumor tissue from a representative patient. In the uninvolved colonic mucosa (en face section), positive ST6Gal-I staining was observed in only a limited number of cells within the crypts. Longitudinal sections of the uninvolved colon tissue (Fig. 3B) revealed that the positively stained cells were localized to the base of the crypts. The staining was focal and located adjacent to the nucleus, typical of Golgi structure. However, the tumor samples demonstrated a dramatic upregulation in ST6Gal-I expression, with punctate-like staining apparent in the majority of the epithelial cancer cells. This type of punctate staining is characteristic of the disrupted Golgi architecture known to be present in cancer cells (20, 21). All seven of the patients examined by immunohistochemistry exhibited the same type of staining pattern shown in Figures 3A & B.

Along with highly upregulated ST6Gal-I expression within the colon tumor tissues, we observed an interesting pattern within several tumor samples; ST6Gal-I appeared to be upregulated in areas of normal appearing crypt structures immediately adjacent to the tumor. Within the malignant region of the tissue sample (Fig. 3Ca), the crypt structure was highly disrupted and ST6Gal-I was overexpressed, similar to results shown in Figure 3A. However, in the morphologically normal-appearing crypts next to the tumor (Fig. 3Cb), ST6Gal-I staining was increased, and distributed in a punctate
pattern, similar to staining in cancer cells. In the crypts more distal to the malignant tissue (Fig. 3Cc), ST6Gal-I expression was very low or undetectable, similar to the uninvolved pair-matched specimens. The upregulation of ST6Gal-I in crypts that appear morphologically intact is reminiscent of a “field effect” in which normal-appearing epithelium is in fact the product of expansion of a genetically abnormal clone.

**ST6Gal-I overexpression in multiple epithelial, but not non-epithelial, tumors**

In addition to colon carcinoma, we examined ST6Gal-I protein expression in several other types of tumors. As shown in Figure 4A, immunohistochemical staining performed on a frozen multi-tissue array revealed ST6Gal-I upregulation in ovarian, stomach, pancreatic and prostate tumors as compared to pair-matched uninvolved tissues. In contrast, ST6Gal-I levels were low or undetectable in malignant and pair-matched uninvolved tissues from brain and skeletal muscle (Fig. 4B).

**ST6Gal-I expression localizes to the stem or progenitor cell compartment in epithelia**

The localization of ST6Gal-I within the base of crypts in non-malignant colon epithelium suggested that ST6Gal-I may be selectively expressed in the stem or progenitor compartment. It is well established that stem and progenitor cells reside in the base of the crypt of normal colon (reviewed in (22). Additionally, the ST6Gal-I staining was very similar to what has been reported for the ALDH1 stem cell marker in normal colon (23). We therefore stained sections of normal human colon (from cancer-free patients) with either ALDH1 or ST6Gal-I. As shown in Figures 5A and B, both the ST6Gal-I and ALDH1 staining were in the base of the crypt, isolated to only a few cells
within each crypt. No detectable staining of ST6Gal-I was observed in the differentiated colonocytes at the apical epithelial surface.

We next examined ST6Gal-I expression in the epidermis, which has clearly defined stem cell compartments (24). One of the compartments for epidermal stem/progenitor cells is the basal epidermal cell layer, immediately adjacent to the basement membrane. As basal epidermal cells differentiate, they migrate apically and lose the capacity for proliferation. As shown in Figure 5C, ST6Gal-I expression was restricted to this basal layer, consistent with the concept that ST6Gal-I may be enriched in stem and/or progenitor cells.

*ST6Gal-I is highly expressed in human induced pluripotent stem cells*

To further explore a possible link between ST6Gal-I and a stem cell phenotype, we evaluated ST6Gal-I levels in iPS cells. Lysates from human iPS cells derived from human foreskin fibroblasts (HFF) and HFF cell lysates were purchased from System Biosciences (SBI, Mountain View, CA). These lysates were immunoblotted for ST6Gal-I expression. We found ST6Gal-I to be highly expressed in iPS cells, with no detectable expression in the HFF cells (Fig. 5D). These results are in agreement with prior microarray results showing ST6Gal-I mRNA to be upregulated in iPS cells, and then downregulated upon forced differentiation of these same cells (25).

*ST6Gal-I expression correlates with stem cell enrichment in colon carcinoma cell lines*

Based on the ST6Gal-I localization in normal and tumor tissues, we evaluated whether ST6Gal-I might be a marker for cancer stem cells. ALDH1 is one of the well-
studied markers for both normal and cancer stem cells (23), and furthermore, immunohistochemical analyses revealed a similar staining pattern for ALDH1 and ST6Gal-I. Accordingly, we examined whether ST6Gal-I expression was associated with the level of stem-cell enrichment in colon cancer cell lines. Our group has generated the human colon carcinoma cell line, HD3, which overexpresses ST6Gal-I secondary to forced oncogenic *ras* expression (18). This cell line was previously transduced with shRNA against ST6Gal-I to obtain a cell population with stable ST6Gal-I knockdown (8). Parental and ST6Gal-I knockdown cells were analyzed for CSC enrichment by flow cytometry using the ALDH1 activity assay, Aldefluor (Stem Cell Technologies, Inc). As shown in Figure 6A, in three independent experiments cells with high ST6Gal-I expression (HD3.par) exhibited significantly greater stem cell enrichment than cells in which ST6Gal-I had been knocked down (HD3.sh). Figure 6B shows a representative dot plot (Run #1, 6A) from this analysis. Cells were also double-labeled for ALDH1 and an additional CSC marker, CD133. As shown in Figure 6C, cells with high endogenous ST6Gal-I expression had significantly greater numbers of cells positive for CD133/ALDH1. These data suggest that forced downregulation of ST6Gal-I significantly decreases the number of CSCs within cancer cell populations.

One important characteristic of CSCs is the capacity to survive traditional chemotherapy treatment (26-28). To study this cellular behavior, we established a cell line with acquired resistance to the camptothecin analog, Irinotecan (CPT-11), a drug used to treat colorectal carcinoma. SW948 colon carcinoma cells were treated with a high dose of CPT-11 (2-fold the IC$_{50}$ dose), and then cultured in stepwise increasing dosages to attain a cell line with >10-fold the IC$_{50}$ dosage of parental cells, resulting in a
stable chemoresistant cell line. Both the parental (SW948.par) and CPT-11-resistant (SW948.CPT) cells were assayed for ALDH1 activity by Aldefluor assay. As shown in Figure 6E, three independent experiments demonstrated that the chemoresistant cells had significant enrichment of ALDH1 activity. Figure 6F is a representative dot plot (Run #1, 6E) from Aldefluor labeling. We next assessed stem cell enrichment by double-labeling cells with anti-CD133 and Aldefluor, which revealed significantly greater numbers of CD133+/ALDH1+ cells in the SW948.CPT cells compared with SW948.par cells (Fig. 6G). We then evaluated ST6Gal-I expression in SW948.par and SW948.CPT cells by immunoblotting cell lysates. Figure 6H shows an acquired ST6Gal-I expression in the established chemoresistant cells. Taken together, these data demonstrate a correlation between CSC enrichment and ST6Gal-I expression in two independent cell model systems. Forced ST6Gal-I downregulation decreases CSC number, whereas acquired chemoresistance yields higher CSC numbers with a corresponding increase in ST6Gal-I expression.

Discussion

Studies over the last two decades have reported increased ST6Gal-I mRNA in many human cancers (reviewed in (1, 2)), and more recent gene expression profiling technologies confirm tumor-associated ST6Gal-I upregulation (29-31). Microarray performed on colon cancer cells isolated by laser capture microdissection revealed higher ST6Gal-I mRNA in tumors with high vs low risk of recurrence (and cells from both tumor types had higher ST6Gal-I than normal colonocytes) (32). Additional microarray studies indicate that ST6Gal-I is overexpressed in cervical (29), testicular (30) and
pancreatic (31) cancers, and ST6Gal-I levels are higher in metastatic vs primary prostate cancer (33). As well, ST6Gal-I is one of the genes downregulated by the metastasis suppressor, BRMS1(34). However few investigations have characterized ST6Gal-I protein expression in either cancer or normal tissues due to the prior lack of anti-ST6Gal-I antibodies. In a study utilizing a privately-generated antibody, ST6Gal-I was found to be upregulated in the majority of human colon tumors (35). In the present study we screened multiple new commercial antibodies and identified a reagent with high specificity for ST6Gal-I. Using this antibody we observed extensive staining for ST6Gal-I in all of the human tumor tissues evaluated by immunohistochemistry, and markedly elevated ST6Gal-I expression in seven out of eight colon tumor samples examined by immunoblotting. Interestingly, the localization of ST6Gal-I in normal tissues was distinctly different from that of tumor tissues. Specifically, ST6Gal-I expression was found within a few cells in the base of the colonic crypts, with no detectable expression in the differentiated epithelial cells. Furthermore, ST6Gal-I expression was high in the basal, proliferative compartment of the epidermis, and high in iPS cells, but undetectable in the somatic cell population from which IPS cells were derived.

Given that ST6Gal-I expression in normal tissues appeared to associate with stem/progenitor cell populations, we evaluated whether ST6Gal-I levels might be elevated in CSCs. CSCs (alternately referred to as “tumor-initiating cells”), are posited to represent a subset of cells within the heterogeneous tumor that has a more aggressive and chemoresistant phenotype (reviewed in (36, 37). The level of CSC enrichment within a cancer cell population is identified by a variety of markers, including ALDH1
and CD133, which have been validated in colon carcinoma (23, 38, 39). CSCs are considered to be a driving force behind tumor reoccurrence due to the self-renewal properties of these cells, and resistance to traditional chemotherapeutic drugs. This has been shown in a variety of cancer types including breast, ovarian and colon carcinomas (26-28). In this study, we found that high ST6Gal-I expression consistently correlated with ALDH1 and CD133 expression, and forced ST6Gal-I downregulation reduced the percentage of CSCs within a heterogeneous cell population. As well, when SW948 colon cancer cells, which do not usually express ST6Gal-I, were treated serially with increasing concentrations of Irinotecan (CPT11), the stem-cell population was selectively protected, evidenced by an increase in ALDH1/CD133-positive cells, and correspondingly, ST6Gal-I expression was markedly increased. Notably, microarray studies comparing gene expression in CD133\(^+\) vs CD133\(^-\) colon cancer cells identified ST6Gal-I as one of the 39 genes with the highest selective expression in CD133\(^+\) cells, and ST6Gal-I was the only glycosylation-related gene in this pool (40). While further studies are needed, these results suggest that ST6Gal-I may represent a new marker for CSCs.

There are several hypotheses concerning the origin of CSCs. It is widely debated as to whether CSCs are derived from mutated normal stem cells, progenitor cells, or differentiated cells (that subsequently revert to a less-differentiated phenotype). In colon tumorigenesis, it has been suggested that a tumor would more likely arise from a mutated stem or progenitor cell, due to the short half-life of differentiated colonocytes, as well as the clonal nature of crypt development, where the entire crypt is thought to be derived from a single stem cell or stem cell compartment located at the base of the crypt (22, 41). Intriguingly, some of the fundamental evidence supporting the clonal crypt hypothesis
was obtained from studies of a sialic acid variant, 9-O-acetylated sialic acid, which is generated by the enzyme, sialate-O-acetyltransferase (OAT). Loss of heterozygosity in stem cells of humans heterozygous for the *OAT* gene causes complete repopulation of the crypt by the progeny of the mutant stem cells (42). While the relationship between 9-O-acetylated sialic acids and ST6Gal-I activity is currently unclear, these studies are consistent with the concept that specific types of sialylation may be very important in maintaining some aspect of the stem cell phenotype.

ST6Gal-I-mediated receptor sialylation has been previously correlated with an undifferentiated or immature cell state, particularly in immune cells. We reported that ST6Gal-I expression is decreased as monocytic cells differentiate down the macrophage lineage (43, 44), and others have shown that ST6Gal-I regulates monocyte-derived dendritic cell generation, and ST6Gal-I expression decreases upon dendritic cell maturation (45). In a separate study, Crespo *et al.* demonstrated that removal of sialic acids via neuraminidase treatment stimulated dendritic cell differentiation, and dendritic cells from ST6Gal-I null mice show a more mature status than cells from wild type mice (46). Also, ST6Gal-I has been shown to be extensively downregulated in activated murine CD4+ and CD8+ T lymphocytes (47). Fewer studies have addressed ST6Gal-I expression in epithelial cell differentiation, however Gabius’ group determined that SNA labeling in epidermis is inversely correlated with differentiation status (48). Finally, Varki and colleagues investigated the role of ST6Gal-I in the PyMT spontaneous mammary tumorigenesis model, and found that tumors from ST6Gal-I null mice were more differentiated than tumors from wild type mice (49).
The functional contribution of ST6Gal-I to an immature or undifferentiated cell phenotype has yet to be elucidated, however resistance to apoptosis may play a prominent role. Accumulating evidence points to ST6Gal-I as a major inhibitor of cell death pathways initiated by Fas, TNFR1 and galectins (2, 11, 12). Lee’s group has also demonstrated that ST6Gal-I confers increased radiation-resistance in colon cancer cell lines (50). In the aggregate, these studies are consistent with the general concept that ST6Gal-I activity might underlie the survival or self-renewal characteristics of stem/progenitor cells, and/or selected cancer cell populations. A corollary hypothesis is that downregulation of ST6Gal-I in differentiated cells may sensitize cells to multiple apoptotic stimuli, thus limiting the lifespan of these cells. Clearly there is a need for further investigation of ST6Gal-I function, however the current study provides important new insight into the localization of ST6Gal-I expression in normal and tumor epithelium, and also implicates ST6Gal-I as a potential new marker for CSCs.

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References


Figure 1: ST6Gal-I is upregulated in human colon tumors. 
(A) Commercially purchased membrane for immunoblotting was probed for ST6Gal-I protein expression. Mature ST6Gal-I (upper band) is highly expressed in three separate colon tumors as compared to normal colon and placenta. The lower band likely represents a cleaved, secreted form of ST6Gal-I. (B) Tissue homogenates were prepared from colon tumors and pair-matched uninvolved colon tissues and immunoblotted for ST6Gal-I. ST6Gal-I was upregulated in 4/5 of colon tumors as compared to pair-matched uninvolved colon tissues. β-actin was used as loading control. U=uninvolved, T=tumor.
Figure 2: Fas receptor has elevated α2-6 sialylation in colon tumors with upregulated ST6Gal-I.

(A) α2-6 sialylated proteins were isolated using SNA1-agarose and immunoblotted for Fas. Total Fas levels were assessed by immunoblotting initial tissue homogenates (not subjected to SNA) for Fas. In all three patient samples, mature Fas (upper band) was downregulated in the tumor tissue relative to the respective uninvolved tissue. In patients with upregulated ST6Gal-I (4&5), the proportion of sialylated Fas to total Fas was 5.07 and 2.55 respectively (densitometry was performed on the upper band). In the patient without upregulation of ST6Gal-I (patient 8), the proportion of sialylated Fas to total Fas was much lower at 1.26. (B) Graphical representation of the ratio of sialylated Fas to total Fas in normal and tumor tissue. U=uninvolved, T=tumor
Figure 3: ST6Gal-I upregulation and localization in human colon tumor samples. (A) Representative sample of pair-matched tissues stained for ST6Gal-I protein expression. Paraffin embedded samples of uninvolved colon tissue and tumor tissues were immunohistologically stained for ST6Gal-I (red), and counterstained with hematoxylin (blue). ST6Gal-I was highly upregulated in tumor tissue, whereas in uninvolved colon tissue, expression was restricted to very few cells within each crypt structure. (B) Longitudinal view of a crypt from uninvolved tissue. ST6Gal-I staining (red) was restricted to the base of the crypt (black arrow). Inset shows enlarged view with ST6Gal-I stain in cells at the base of the crypt. (C) ST6Gal-I staining in a patient sample showed gradient expression based on proximity to tumor. (a) upregulated expression of ST6Gal-I in malignant tissue, (b) aberrant expression in seemingly normal crypt structures directly adjacent to tumor and (c) low expression in crypts distal to the tumor.
Figure 4: ST6Gal-I is upregulated in several types of epithelial cancers, but not non-epithelial cancers.
(A) Frozen epithelial tumors and pair-matched uninvolved tissues from ovary, pancreas, stomach, and prostate were stained for ST6Gal-I protein expression (red) and counterstained with hematoxylin (blue). ST6Gal-I upregulation was apparent in the tumor samples. (B) Frozen pair-matched tissues from skeletal muscle and brain exhibited low or undetectable levels of ST6Gal-I.
Figure 5: ST6Gal-I expression in stem/proliferative cell populations.
(A) Paraffin-embedded normal colon tissue (from a cancer-free patient) was stained for ST6Gal-I. ST6Gal-I (red stain) expression was confined to the base of normal colon crypts, with no expression observed in the apical, differentiated epithelium. (B) Expression of the ALDH1 stem cell marker was localized to the base of the crypts in normal human colon, similar to ST6Gal-I. (C) Staining for ST6Gal-I (brown stain) in paraffin-embedded normal human skin tissue. ST6Gal-I expression was confined to the basal proliferative compartment of the epidermis in normal human skin tissue. (D) Immunoblot for ST6Gal-I expression in human induced Pluripotent Stem cell (iPSc) lysate and the human foreskin fibroblast (HFF) cell lysate from which the iPSc cells were derived. There was no detectable ST6Gal-I expression in HFF cells whereas there was a dramatic upregulation of ST6Gal-I in the iPSc cells. β-actin was used as a loading control.
Figure 6: ST6Gal-I expression correlated with cancer stem cell enrichment.
(A) Colon carcinoma cells, HD3.par and HD3.sh, were assayed for ALDH1 activity (Aldefluor) by flow cytometry. Enrichment of ALDH1 staining was significantly higher in HD3.par as compared to HD3.sh in three independent runs. (B) Representative dot plot (run #1, 6A) showing ALDH1 staining. (C) Double labeling for stem cell enrichment of HD3.par and HD3.sh cells with ALDH1 and CD133 by flow cytometry revealed that knockdown of ST6Gal-I lead to significantly decreased enrichment in three independent runs. (D) Immunoblot of HD3.par and HD3.sh cells showed that shRNA transduction reduced ST6Gal-I expression. (E) ALDH1 activity was assayed by flow cytometry in colon carcinoma cell line SW948. SW948.CPT chemoresistant line had significant enrichment for ALDH1 staining in three independent runs as compared to SW948.par. (F) Representative dot plot of ALDH1 staining (run #1, 6E). (G) Double-labeling of SW948.par and SW948.CPT with ALDH1 and CD133 showed significant increase in stem cell markers in the chemoresistant line (SW948.CPT) in three independent runs. (H) Immunoblot of SW948.par and SW948.CPT shows ST6Gal-I expression was upregulated in the SW948.CPT line. β-actin was used as a loading control. *=P<0.001.
Supplementary Figure 1: Validation of ST6Gal-I antibody.
(A) Two cell lines, SW48 colon cancer cells and OV4 ovarian cancer cells, both lacking endogenous ST6Gal-I expression, were forced to express ST6Gal-I by lentiviral transduction (note that SW48 and SW948 are two distinct cell lines). Lysates from these lines were immunoblotted for ST6Gal-I, which showed antibody binding to a 50kD protein present only in the ST6Gal-I expressing cells (EV = empty vector). (B) OV4 cells, both parental cells and cells forced to express ST6Gal-I, were immunofluorescently stained for ST6Gal-I (green). Nuclei were stained with Hoescht (blue). Positive antibody staining was restricted to the ST6Gal-I-expressing cells, and was localized in a focal region near the nucleus, characteristic of Golgi structure. (C) OV4 cells stained by immunohistochemistry showed apparent Golgi-localized ST6Gal-I expression in cells with forced ST6Gal-I.
Supplementary Figure 2: Negative controls for ST6Gal-I staining show no nonspecific staining.

(A) Frozen slides containing colon metastasis to liver were subjected to incubation with ST6Gal-I antibody or isotype control IgG (Sigma). Signal was developed with NovaRed, then counter-stained with hematoxylin to detect non-specific labeling. There was no detectable non-specific labeling in the isotype IgG control (red stain). (B) Paraffin-embedded pair-matched colon tumor and uninvolved colon were exposed to secondary antibody alone (no primary antibody). There was no detectable staining in either section.
DISCUSSION

Cancer-associated changes in glycosylation have been established in
tumorigenesis for over twenty years and many of the endogenous anti-tumor antibodies
produced by patients are targeted to carbohydrate antigens (138-140). However, progress
in this area of research has lagged behind other areas due to the technical challenges that
plague the field of glycobiology research. Unlike the predictable synthesis of proteins
and oligonucleotides, the synthesis of glycoconjugates is not readily projected, and aside
from identifying possible targets within peptide sequences, it is difficult to predict and
characterize glycan elaboration. While consensus sequences targeted for glycan addition,
such as the N-x-S/T sequence for N-linked glycans, may be available within a protein, it
is important to clarify that not every target sequence receives a glycan. The factors
affecting this specificity are still unclear, and this presents an area of research for future
work. We do know that specificity of targets is important in cell behavior, including
migration and invasion. Our laboratory has shown ST6Gal-I to terminally sialylate N-
glycans on the β1, but not β3 or β5, integrins, and this specificity drives tumor-promoting
phenotypes, such as increased migration and invasion mediated by β1 integrin binding to
collagen-1 (23, 25, 43, 49-51). However, what makes one protein a target while the
others may not be is currently unknown. Despite the lack of clarity as to what drives the
specificity and characteristics of protein glycosylation, our laboratory has used
genetically manipulated cell lines to focus on identifying protein targets for ST6Gal-I
sialylation, and determining how α2-6 sialylation may impact the function of targeted proteins.

The focus of the first section of this dissertation was to explore the role of ST6Gal-I in a specific manner by identifying what effects sialylation had on the function of the Fas death receptor in regulating tumor cell survival. Previous research from our laboratory was centered on integrin-mediated modes of survival/apoptotic signaling, and we have shown that ST6Gal-I sialylation of the β1 integrin protects cells against galectin-3 mediated apoptosis (62). Tumors have been shown to upregulate galectin expression and intracellular galectins act in protumorigenic signaling; however, extracellular galectin binding induces apoptosis (141). By using the forced expression cell model, SW48, we were able to determine that ST6Gal-I specifically inhibited extracellular galectin-3 mediated apoptosis by blocking binding of galectin-3 to β1 integrins. This study shed light on a role for ST6Gal-I in protective mechanisms against apoptosis, and with that in mind, we began to look at other mechanisms of apoptotic signaling aside from integrin-mediated signaling. Here, we have identified Fas as a specific target for ST6Gal-I, and demonstrated that sialylation of Fas was a mechanism through which cancer cells were protected against apoptotic signaling. This work also demonstrated that sialylation by ST6Gal-I does not protect against DR4/DR5 apoptotic signaling induced by the endogenous ligand for these receptors, TRAIL. By using two distinct cell models, both a forced expression (SW48) and a knockdown (HD3), we have shown by direct manipulation of ST6Gal-I that sialylation of Fas is a mechanism of protection against apoptosis. More specifically, treatment with both the activating antibody (CH11) and the endogenous ligand for Fas (FasL) resulted in decreased apoptotic signaling in cells
expressing ST6Gal-I. We found sialylation of Fas blocks apoptotic signaling by inhibiting DISC formation and blocking internalization.

Significance of Attenuated Fas Internalization

The potential significance of this finding is two-fold. First, one of the defining characteristics of the tumor cell is the ability to evade cell death (142). We have identified Fas as a target for ST6Gal-I sialylation, and have shown that this sialylation protects cells from Fas-mediated apoptosis. This protective mechanism further identifies ST6Gal-I as a regulator of cell death mechanisms. In addition to protecting against galectin-3 and Fas-mediated apoptosis, our laboratory has shown the death receptor TNFR1 to be a target for ST6Gal-I sialylation in monocytes (60). This sialylation confers protection against apoptosis induced by the endogenous ligand, TNFα. We found that sialylation reduces the amount of TNFα-induced apoptosis, and this effect can be reversed by either enzymatically removing sialic acids or by knocking down ST6Gal-I expression. In addition to protection against TNFR1-mediated apoptosis, we also found that primary macrophages from an ST6Gal-I overexpressing transgenic mouse model created by our lab demonstrate protection against Fas-mediated apoptosis induced by the Fas-activating antibody Jo-2.

Although the study of ST6Gal-I protection against TNFR1-mediated apoptosis was conducted in monocytes, TNFR1 is expressed on epithelial cell surfaces, and should be examined in the future as an additional avenue of protection against apoptosis in epithelial tumors. Furthermore, while this dissertation has focused heavily on tumor cell behavior after death receptor activation, an area of research that would most likely yield
valuable information about these mechanisms is the interactions of tumor cells with the immune environment, as immune cells are most likely a major supplier of the ligands necessary to activate these signaling pathways (i.e. TNFα, FASL, galectin-3).

Recent reports from our laboratory and others have demonstrated ST6Gal-I mediated protection against a milieu of cell death stimuli, ranging from endogenous mechanisms of apoptosis induced by galectin-3, FasL, and TNFα, to more clinically relevant stimuli such as chemotherapeutics and radiation treatments. In one study, Lee’s group identified EGFR as a substrate for ST6Gal-I sialylation, and demonstrated that knockdown of ST6Gal-I increased susceptibility to cell death induced by the EGFR kinase inhibitor gefitinib in three independent colon carcinoma cell lines. Additionally, they showed forced expression of ST6Gal-I to protect against gefitinib-induced apoptosis in a cell line lacking endogenous ST6Gal-I (143). This group also has shown ST6Gal-I forced expression to protect against radiation-induced apoptosis in colon carcinoma cell lines (144). Unpublished work from our laboratory also suggests ST6Gal-I confers protection against other chemotherapy reagents, including cisplatin. Interestingly, studies have shown Fas signaling to play a role in cisplatin-induced killing of cancer cells (145, 146). This presents an interesting possibility that one potential mechanism of ST6Gal-I protection against cisplatin-mediated apoptosis is through altered signaling of sialylated Fas. Altogether these studies highlight an important translational aspect of ST6Gal-I protection by showing protection against not only endogenous apoptotic mechanisms but also against treatment-induced apoptosis important in cancer therapies.

Along with protection against apoptosis, upregulation of ST6Gal-I and resulting hypersialylation of targets may provide a mechanism for signaling for other tumor-
promoting activities. A main goal of this work has been to demonstrate ST6Gal-I protects against Fas-mediated apoptosis through the prevention of internalization. The effects of this altered internalization could be two-fold. The first is inhibition of apoptosis as discussed earlier, while the second side of this altered Fas signaling story may hinge on the Fas receptor working as more than a traditional “death receptor.” Recent literature has begun to point to Fas as not only an inducer of apoptosis, but also having an imperative role in other tumor phenotypes including tumor cell survival, proliferation, migration and invasion (100, 118-121). Work from Marcus Peter’s laboratory demonstrates that Fas signaling is necessary for tumorigenesis. In this study, proliferation of several cell lines is significantly inhibited by knocking down Fas expression. Moreover, deletion of Fas receptor prevents tumorigenesis in an in vivo spontaneous model of ovarian cancer and decreases liver tumor formation (117). Interestingly, literature suggests these alternate roles may be dependent on Fas localization on the cell surface (100). Studies have shown internalization of Fas after ligand binding leads to high levels of DISC formation, caspase cleavage, and apoptosis. However, Fas remaining on the cell surface after activation can lead to alternative signaling cascades, including those activating cell survival or proliferation (100). Preliminary work from our laboratory suggests ST6Gal-I sialylation of the Fas receptor may play a role in regulating some of these alternate signaling cascades. This suggests ST6Gal-I sialylation of the Fas receptor may act as a switch between the pro-apoptotic signaling mechanisms and the pro-tumorigenic signals by working to maintain the Fas receptor at the cell surface (Figure 6).
Relevant to the previous discussion of sialylation-induced apoptosis resistance, Ametller et al. have recently shown that Fas has tumor-promoting roles in oxaliplatin-resistant colon carcinoma cell lines (119). In this work, oxaliplatin treatment stimulated tumor cell migration through Fas activation, an effect that was inhibited by knockdown of Fas. While the link between sialylation of Fas, chemoresistance, and alternative Fas signaling is yet to be determined, it is interesting to consider that this mechanism of Fas-migration may rely on ST6Gal-I sialylation of the Fas receptor by protecting against apoptotic signaling in order to preserve alternative Fas signaling mechanisms (Figure 6).

The mechanisms attenuating internalization are yet to be determined. However, when examining ST6Gal-I sialylation as a regulator of the tenure of Fas protein at the surface, it is important to consider that the sialic acid addition adds a negative charge to the protein, contributing to a variety of changes similar to phosphorylation. With this approach, it may be speculated that the addition of the negatively charged sugar could alter Fas internalization by a variety of means such as: 1) changes in tertiary protein conformation, 2) altered Fas homotrimeric or higher-order clustering, or 3) lipid-raft localization. We have shown ST6Gal-I sialylation of the β1 integrin alters its conformation (23). Also, work from Baum’s group showed sialylation of CD45 disrupted clustering of the receptor, and thereby altered internalization (147). Additionally, lipid rafts carry a negative charge, which may present electrostatic repulsion to sialylated Fas receptors. The mechanistic details of this attenuated internalization should be pursued further.
ST6Gal-I Expression and Fas Sialylation in Human Tissues

The full scope of the effects of ST6Gal-I sialylation of the Fas receptor is not yet known. We do know Fas receptor is sialylated in human tumors, as has been shown in the second section of this dissertation. By examining Fas expression and relative Fas sialylation, we found in patient tumors with ST6Gal-I upregulation, the Fas receptor carried an increased proportion of sialylation as compared to uninvolved colon tissue from the same patient. Hypersialylated Fas receptors within tumor samples demonstrate that not only is the enzyme upregulated, but that it is hypersialylating targets in an in vivo environment. This further confirms work published previously by our lab in which 100% of human tumors assayed contained hypersialylated β1 integrin (49). These patient studies bring to the forefront an area of research yet to be fully developed. Studies attempting to link ST6Gal-I expression and sialylation with patient survival, treatment response, and metastasis occurrence may shed light on the regulation of Fas signaling through ST6Gal-I sialylation. This may have important implications for not only basic tumorigenic mechanisms (i.e. proliferation and evasion of apoptosis) but also metastasis and chemoresistance.

These studies are now feasible in part due to the work laid out in the second section of this dissertation. While the first section of this dissertation was focused on molecular mechanisms of ST6Gal-I sialylation and effects on Fas signaling, the second section focused more on the identification of ST6Gal-I localization within human tissues. Historically, the field has relied heavily on SNA labeling and mRNA analysis for detailing ST6Gal-I expression within human tissues. Now, due to the validation a
commercially available antibody, ST6Gal-I protein expression can be characterized in a much more efficient and reliable fashion.

We have also identified ST6Gal-I localization in a variety of human tissues, including several samples of colon carcinoma and uninvolved colon tissue. We found ST6Gal-I to be highly upregulated in several epithelial cancers, including colon, pancreas, prostate, stomach and ovarian carcinomas. Increased levels of ST6Gal-I were expected in tumor samples, due in part to the upregulation of ST6Gal-I transcripts driven by oncogenic-ras. However, we unexpectedly found that ST6Gal-I differentially localizes to the base of the crypt in normal human colon samples, and the basal layer of normal epidermis. While ST6Gal-I expression has been loosely correlated with an undifferentiated cellular status, this is the first study to show localization to the base of normal colon crypts, with striking similarity to the known normal stem cell marker ALDH1. Furthermore, we found ST6Gal-I expression to correlate with enrichment of the cancer stem cell markers ALDH1 and CD133 as knockdown of ST6Gal-I decreased ALDH1+ and ALDH1+/CD133+ double labeling. While these studies show an association of ST6Gal-I expression with stem-like populations, further investigations into whether ST6Gal-I is a regulator of this stem cell-enriched phenotype are needed.

In addition, ST6Gal-I correlated with the ALDH1+/CD133+ double-labeled population in a colon carcinoma cell line selected for chemoresistance. In this study, SW948 cells selected for resistance to the chemotherapeutic CPT-11 (Irinotecan) demonstrated increased stem cell enrichment as compared to the chemosensitive parental line from which it was derived. The chemoresistant line also had highly upregulated ST6Gal-I. The mechanism behind this upregulation is yet to be determined. However,
this study demonstrating increased ST6Gal-I in a CPT-11-resistant population combined with the studies showing ST6Gal-I conferred resistance to both cisplatin and gefitinib presents three independent chemotherapeutic pathways in which ST6Gal-I may play a role in chemoresistance. Decreased susceptibility due to ST6Gal-I expression most likely presents only one of many mechanistic details pertaining to susceptibility to or resistance against chemotherapeutics. However, this concept may have a more translational application, as ST6Gal-I expression could be a possible biomarker for chemoresistance, and presents an exciting avenue for future research.

Here we have presented a novel role for ST6Gal-I as a modulator of Fas receptor signaling. We have also provided an unanticipated departure from our previous work with the correlation of ST6Gal-I with stem cell phenotypes, including normal colon, epidermis, and cancer stem cell enrichment. While this was not an expected result, it is not too vast of a conceptual leap considering the effects from ST6Gal-I expression as a whole. While the phenotypic results of ST6Gal-I expression such as decreased differentiation, increased migration, and now the ability to avoid cell death have been demonstrated using cancer models, these are also classic phenotypes of a stem or progenitor cell. The finding that ST6Gal-I is upregulated not only in cancer but also associated with stem cell niches (colon and skin) underscores the importance of further investigation into the role of ST6Gal-I in normal tissue and initial tumorigenesis.

ST6Gal-I and the Stem Cell Hypothesis

One impact that a possible correlation between ST6Gal-I expression and stemness may have is to shed light onto the origin of cancer stem cells. Several hypotheses are
currently being debated including proposals that cancer stem cells may arise from mutated normal stem cells, progenitor cells, or differentiated cells. Concerning colon tumorigenesis, it has been suggested that it may be more likely that a tumor would occur from a mutated progenitor or normal stem cell (33). This is due in part to the fact that differentiated colonocytes have a relatively short half-life and these cells are too short-lived to acquire and propagate a malignant phenotype. Additionally, successive replication of the colonic stem cells may provide increased opportunity for oncogenic mutations, which would then be passed on to the daughter cells. The clonal nature of crypt development in which the entire crypt is thought to be derived from a single stem cell or stem cell compartment located at the base of the crypt provides further support for this hypothesis (33). Therefore, it could be postulated that a mutated stem cell could repopulate a previously normal crypt with mutated, tumorigenic cells (33, 148).

Interestingly, some of the more compelling data supporting the clonal crypt hypothesis are a group of studies that focuses on tracking a chemical variant of sialic acid within the colonic crypts of normal and FAP patients. These studies trace the presence of a sialic acid variant, 9-O-acetylated sialic acid, showing that loss of heterozygosity in the stem cells of these patients lead to repopulation throughout the affected crypt. While the relationship between ST6Gal-I and these studies is yet to be determined, it is interesting that the most reliable evidence of clonal crypt expansion involves changes in sialic acid variants in the colon epithelium (148-151). In the latter part of this dissertation, we have shown ST6Gal-I protein levels to be highly upregulated in the iPS cells, even to the point of on/off expression, as compared to their parental fibroblast cells. ST6Gal-I staining is correlated with the stem cell marker ALDH1 staining the basal compartment of normal
colon and localized to the basal epidermis in skin. Also, we have seen ST6Gal-I expression to be highly upregulated in colon tumor samples, with it being found in the majority of the epithelial-like tumor cells within each sample, similar to other colon cancer stem cell markers ALDH1 and CD133 (133, 152). More studies are needed to further elucidate the role ST6Gal-I may be playing in the stem cell phenotype. While the origin of cancer stem cells within the colon is a multifaceted and complex question, these data presented here support the idea that colon cancer stem cells may originate from a mutated stem cell, or progenitor cell within the normal colon crypt.

This dissertation presents several novel roles for ST6Gal-I. We have shown that ST6Gal-I sialylation of the Fas receptor confers apoptosis. We have also shown ST6Gal-I protein expression is highly upregulated in colon carcinoma, and that expression is correlated with stem cell markers, ALDH1 and CD133. In sum this work not only answers questions about how ST6Gal-I may regulate tumorigenesis and normal tissue, but also provides an exciting new avenue to be pursued as to the possible role of ST6Gal-I in stem cell biology. Altogether, work presented herein provides evidence that ST6Gal-I contributes to tumorigenesis in two important aspects: the protection against death receptor mediated apoptosis and also the enrichment of cancer stem cell populations. This highlights an interesting possibility that targeting ST6Gal-I therapeutically may provide a mechanism to increase cell death, while decreasing chemo resistance as well as metastatic potential of existing tumors.
Figure 6: Possible Fas Signaling Switch Mechanism
ST6Gal-I sialylation of Fas may act as a switch from apoptosis to other signaling cascades. By blocking the internalization of the Fas receptor after activation, the Fas receptor remains on the cell surface, where it may function to signal through alternative pathways.


6. Schenk B, Fernandez F, Waechter CJ. The ins(ide) and out(side) of dolichyl phosphate biosynthesis and recycling in the endoplasmic reticulum. Glycobiology. 2001;11:61R-70R.


APPENDIX

NOTICE OF APPROVAL: HUMAN USE
DATE: October 11, 2012

MEMORANDUM

TO: Amanda F. Swindall
   Principal Investigator

FROM: Cari Oliver, CIP
      Assistant Director

RE: Request for Determination—Human Subjects Research
    IRB Protocol #N120922009—Role of Receptor Sialylation in the Tumor Cell Phenotype (Glycosylation-Dependent Mechanisms for Ovarian Tumor Cell Resistance to Cell Death)

A member of the Office of the IRB has reviewed your application for Designation of Not Human Subjects Research for above referenced proposal.

The reviewer has determined that this proposal is not subject to FDA regulations and is not Human Subjects Research. Note that any changes to the project should be resubmitted to the Office of the IRB for determination.