

AD _____

Award Number: W81XWH-11-1-0404

TITLE: Role of receptor sialylation in the ovarian tumor cell phenotype

PRINCIPAL INVESTIGATOR: Susan L. Bellis

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham, AL 35294 294

REPORT DATE: June 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 01-06-2012		2. REPORT TYPE Annual report		3. DATES COVERED 15 May 2011 - 14 May 2012	
4. TITLE AND SUBTITLE Role of receptor sialylation in the ovarian tumor cell phenotype				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-11-1-0404	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Susan L. Bellis E-Mail: bellis@uab.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Alabama at Birmingham Birmingham, AL 35294				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The overarching goal of our research is to define the role of a cancer-associated glycosyltransferase, ST6Gal-I, in regulating the ovarian tumor cell phenotype. In the first year of this pilot project, significant progress has been made toward establishing ST6Gal-I's function in protecting tumor cells from apoptosis. These results establish a new paradigm in apoptotic signaling, particularly given that there is a marked dearth of evidence concerning glycosylation-dependent mechanisms in tumor cell survival. We also show that tumor cells can be sensitized to cisplatin-induced cell death through forced downregulation of ST6Gal-I. Platin drugs represent a first-line treatment for ovarian cancer, therefore therapeutic targeting of ST6Gal-I may hold promise for treating patients that have become chemoresistant. Finally, preliminary results indicate that ST6Gal-I protein is upregulated in human ovarian tumors, implicating ST6Gal-I as a potential new biomarker. In the upcoming year, our studies will focus on elucidating the mechanistic basis by which ST6Gal-I modulates the activity of integrins and death receptors to control tumor cell invasion and apoptosis-resistance.					
15. SUBJECT TERMS Chemoresistance, death receptors, apoptosis, glycosylation, invasion, metastasis, galectin					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU	8	19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

INTRODUCTION.....	1
BODY.....	2
KEY RESEARCH ACCOMPLISHMENTS.....	4
REPORTABLE OUTCOMES.....	4
CONCLUSION.....	4
REFERENCES.....	4

INTRODUCTION

The ST6Gal-I sialyltransferase, which adds α 2-6-linked sialic acids to membrane glycoproteins, is up-regulated in multiple cancers, including ovarian carcinoma, and increased ST6Gal-I activity is associated with metastasis and poor patient prognosis. Moreover, both cell culture studies and animal models support a role for ST6Gal-I in metastasis. However, because the substrates for ST6Gal-I have not previously been well-defined, there is little understanding of the mechanism by which ST6Gal-I promotes tumor progression. Our group identified the β 1 integrin as an ST6Gal-I substrate, and showed that elevated integrin sialylation promotes cell migration on collagen I, and invasion through Matrigel. In addition, integrin sialylation blocks apoptosis induced by the mammalian lectin, galectin-3, which our studies show is expressed in human ovarian tumor tissues and in ascitic fluid from patients with metastasis disease. Finally, we have recently identified the Fas and TNFR1 death receptors as ST6Gal-I substrates, and shown that sialylation of these receptors strongly inhibits apoptotic signaling. Collectively our studies suggest that elevated ST6Gal-I expression provides a selective advantage for tumor cells through multiple molecular pathways. The *central hypotheses* of our study are that increased receptor sialylation (secondary to ST6Gal-I upregulation) contributes to the invasive and apoptosis-resistant phenotype of ovarian cancer cells and (2) ovarian cancer progression can be inhibited by therapeutic targeting of ST6Gal-I expression/activity. To address these hypotheses we will pursue the following Specific Aims:

Specific Aim 1: Role of β 1 integrin sialylation in regulating ovarian tumor cell association with the omentum

Specific Aim 2: Determine whether ovarian tumor tissues upregulate gal-3, and whether integrin sialylation protects tumor cells from gal-3-mediated apoptosis

Specific Aim 3: Resistance to apoptosis conferred by ST6Gal-I-mediated sialylation of death receptors

PROGRESS REPORT (body)

Overview of progress:

Although we had initially planned to begin by pursuing Aim 1, we have instead made the most progress on Aim 3. In particular, we have unequivocally shown a role for ST6Gal-I-mediated sialylation in resistance to the chemotherapeutic drug, cisplatin (manuscript in preparation). Our collective results indicate that tumor cells that are resistant to cisplatin have upregulated ST6Gal-I, whereas chemosensitivity to cisplatin can be restored by forced downregulation of ST6Gal-I. These data have clear translational implications related to treatment of chemoresistant tumors, which is a major problem in ovarian cancer patients. In addition to our work on Aim 3, we have made some progress on each of the aims, as outlined below:

Specific Aim 1: Role of the β 1 integrin receptor in regulating ovarian tumor cell association with the omentum.

Within Aim 1, we have made the most progress on Task 3: Cell invasion into murine omental cultures. Murine omenta have been successfully isolated as described by our collaborator [1], and we have shown that these cultures are viable for at least 10 days, and that human cells can adhere to the omental surface. Human ovarian cancer cell lines were seeded onto the omental cultures, and allowed to invade for 24 hours. The samples were then frozen and sectioned, and analyses of cell invasion in these sections are ongoing. To quantitatively measure cell invasion, my laboratory has developed a custom Matlab software program [2] that can track the invasion distance for each cell within the specimens. One challenge that was encountered concerns the use of GFP to visualize cells. Due to the autofluorescence of native tissue, the signal:noise ratio of GFP-labeled cells was high, therefore we have switched our cell labeling technique to a quantum dots method. With this new protocol in place, we are ready to move forward to the next set of experiments, which will focus on the use of function-blocking antibodies or siRNA to determine the role of the β 1 integrin in cell adhesion and invasion into omentum (Task 4). Once studies of cell interactions with murine omental cultures are complete, we will initiate studies of cell interaction with human omental tissues (Tasks 1 and 2). It is anticipated that data generated from the murine cultures will be very useful for guiding studies with human tissues (eg. optimal time points, cell number, cell labeling techniques, etc). Given the need for freshly-harvested human tissue, it seems prudent to take advantage of the murine cultures to optimize the experimental system prior to executing the human omental culture experiments.

Specific Aim 2: Determine whether ovarian tumor tissues upregulate gal-3, and whether integrin sialylation protects tumor cells from gal-3-mediated apoptosis.

Progress on Aim 2 has been primarily centered on collecting human ascites fluid (Task 1). In addition, we have confirmed that gal-3 is expressed in all of the ascites analyzed to date (Task 1b), and that ovarian tumor tissues have upregulated ST6Gal-I (Fig 1). This is the first time immunohisto-

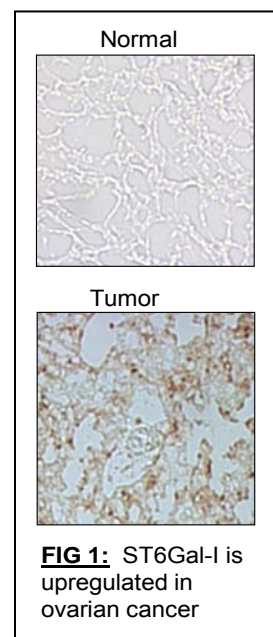
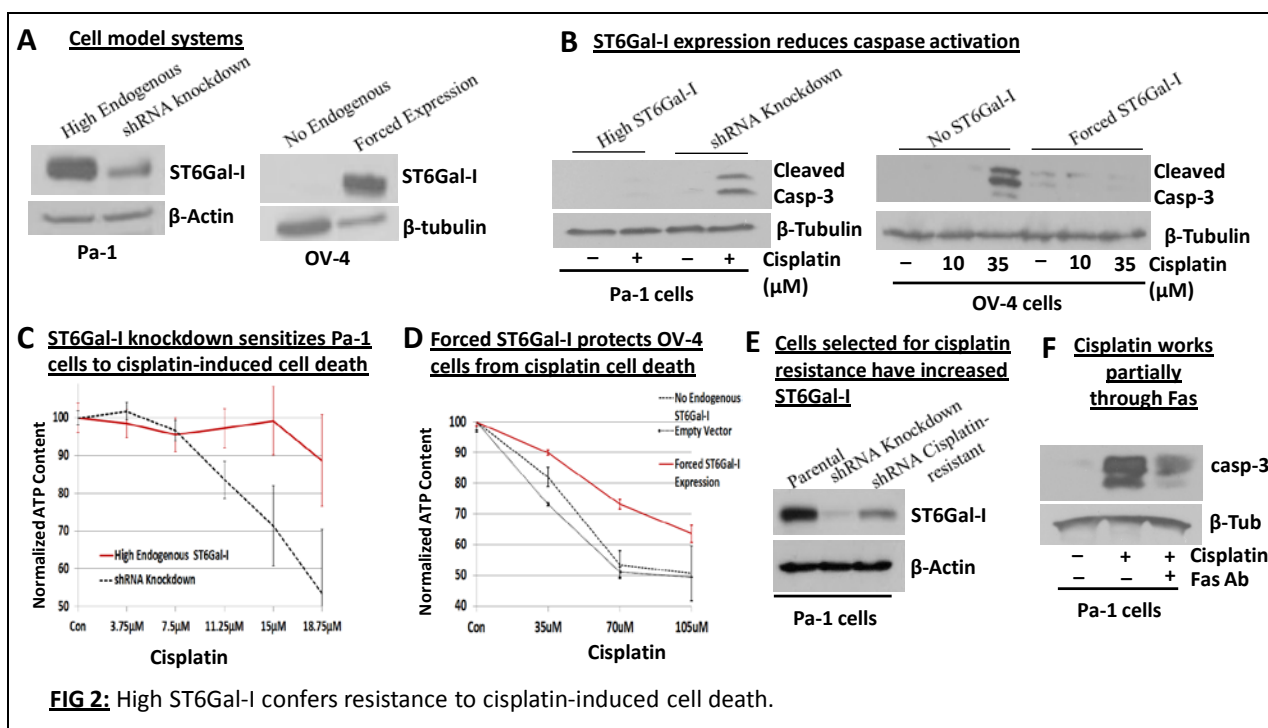


FIG 1: ST6Gal-I is upregulated in ovarian cancer

chemistry for ST6Gal-I has been performed on ovarian tissues, and the first confirmation that ST6Gal-I protein is upregulated in ovarian cancer. In the upcoming year, we will direct more effort toward completion of Aim 2.

Specific Aim 3: Resistance to apoptosis conferred by ST6Gal-I-mediated sialylation of death receptors.

Progress has been made on all of the tasks within this aim. We have determined that ST6Gal-I-mediated sialylation of Fas and TNFR1 blocks apoptotic signaling by preventing receptor internalization and DISC formation (Tasks 1 and 2). We have also shown that cells with high ST6Gal-I expression are resistant to cisplatin-directed apoptosis (Task 3). This was determined in 2 independent cell model systems: (1) forced ST6Gal-I knockdown in Pa-1 cells that have high endogenous ST6Gal-I and (2) forced overexpression of ST6Gal-I in OV-4 cells that have no endogenous ST6Gal-I (Fig 2A). As shown in Fig 2B, ST6Gal-I knockdown in Pa-1 cells enhances caspase activation, whereas ST6Gal-I forced expression in OV-4 cells inhibits caspase activation. Furthermore, ST6Gal-I knockdown in Pa-1 cells decreases cell viability (Fig 2C), and conversely, ST6Gal-I overexpression in OV-4 cells increases cell viability (Fig 2D). To evaluate a physiologic role for ST6Gal-I in chemoresistance, Pa-1 cells with ST6Gal-I knockdown were treated with cisplatin for 3 weeks, and the small number of cells surviving this treatment were collected and expanded in culture. As shown in Fig 2E, cells resistant to apoptosis had elevated expression of endogenous ST6Gal-I, suggesting that ST6Gal-I-expressing cells have a survival advantage. Finally, we pretreated Pa-1 cells with function-blocking antibodies to Fas, and then incubated the cells with cisplatin (Fig 2F). These experiments showed that inhibiting Fas signaling diminished cisplatin-induced caspase 3 activation, implicating Fas in some aspect of cisplatin-dependent apoptosis. In the upcoming year, we will perform mechanistic studies to determine whether sialylation of Fas (vs other receptors) is responsible for the negative effects of ST6Gal-I on cisplatin efficacy. These studies have high potential impact for the use of ST6Gal-I as a possible chemoresistance marker in ovarian cancer



patients, and as a new molecular target for enhancing therapeutic effectiveness of platin drugs.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of viable omental cultures.
- Optimized immunoblotting protocol for galectin-3 in ascites
- Determination that sialylation of Fas and TNFR1 blocks apoptotic signaling by preventing DISC formation and receptor internalization.
- First demonstration that ST6Gal-I protein levels are upregulated in ovarian cancer (all other studies evaluated ST6Gal-I mRNA).
- First report that ST6Gal-I expression confers resistance to cisplatin-mediated apoptosis.

REPORTABLE OUTCOMES

- Abstract and poster presentation for the 2012 annual meeting of the American Association for Cancer Research (abstract appended).
- Development of unique ovarian cancer cell lines with forced overexpression or knockdown of ST6Gal-I.
- Although not a part of the original proposal, we have developed a new overexpressing ST6Gal-I transgenic mouse model that will be ideal for examining the role of ST6Gal-I in spontaneous ovarian cancer.

CONCLUSION

An alteration in the profile of cell surface glycans was one of the earliest identified hallmarks of a tumor cell, however we still know very little regarding the functional contribution of tumor-associated glycoconjugates. The studies described herein provide critical new insights into the role of the ST6Gal-I sialyltransferase in controlling the tumor phenotype. In particular, we have shown that sialylation of selected receptors has a strong inhibitory effect on multiple apoptotic pathways, including death receptor signaling and cisplatin-induced cell death. These findings are important because they establish a strong foundation for pursuing ST6Gal-I as a potential biomarker for ovarian cancer progression, and new molecular target for therapeutic intervention. The identification of new molecular pathways involved in ovarian cancer is essential, because survival rates for ovarian cancer have not changed appreciably for more than two decades. Having made substantial progress in understanding the role of ST6Gal-I in tumor cell death (Aim 3), we will, in the upcoming grant year, increase our efforts toward defining the role of integrin sialylation in invasion (Aim 1) and resistance to galectin-mediated cell death (Aim 2).

REFERENCES

- 1 Khan, S. M., Funk, H. M., Thiollay, S., Lotan, T. L., Hickson, J., Prins, G. S., Drew, A. F. and Rinker-Schaeffer, C. W. Clin Exp Metastasis. **27**, 185-196
- 2 Phipps, M. C., Clem, W. C., Grunda, J. M., Clines, G. A. and Bellis, S. L. Biomaterials. **33**, 524-534

APPENDIX (attached)

Abstract from 2012 annual meeting of the AACR

Altered receptor glycosylation confers cisplatin resistance in ovarian cancer cells

Matthew Schultz, Susan Bellis PhD

Department of Biophysics and Physiology, University of Alabama at Birmingham

Chemotherapy resistance is a significant clinical obstacle in the treatment of cancer. Platinum-based drugs are a mainstay of treatment in many solid cancers; however, tumor resistance to these drugs hampers long term efficacy. Resistance is multi-factorial, and theories to explain resistance are incomplete. In this study we report a novel mechanism for resistance to cisplatin involving changes in receptor glycosylation. Previous work in our lab has demonstrated enhanced colon cancer cell survival in cells expressing the sialyltransferase ST6Gal-I. We showed that sialylation of the Fas death receptor by ST6Gal-I in these cells decreases apoptotic signaling in response to Fas agonists. Elevated ST6Gal-I expression has been reported in many cancers, including ovarian and colon, and its expression is negatively correlated with patient prognosis. ST6Gal-I resides in the golgi and adds a negatively-charged sialic acid to cell-surface and secreted glycoproteins. The enzyme demonstrates substrate specificity, shown by the observation that it sialylates the $\beta 1$ integrin but not $\beta 3$ or $\beta 5$. We have shown that sialylation of the Fas receptor interferes with receptor internalization after stimulation with Fas agonists in colon cancer cells. Interestingly, cisplatin has been demonstrated to mediate its cytotoxic effects, in part, through Fas receptor upregulation and activation, although the mechanism is not currently understood. Combining these results and observations, we investigated whether ST6Gal-I-mediated sialylation of the Fas receptor inhibits apoptotic signaling in response to cisplatin treatment, contributing to intrinsic cisplatin resistance.

We determined the extent of cisplatin-induced apoptotic cell death by blotting for cleaved caspase-3, a terminal caspase, and detected decreased levels of activated caspase in ST6Gal-I expressing ovarian and colon cell lines. This trend was observed in both a low endogenous ST6Gal-I cell model with forced ST6Gal-I expression, as well as a high endogenous ST6Gal-I expression cell model with shRNA ST6Gal-I knockdown. We also show sustained viability in cells expressing ST6Gal-I after cisplatin treatment by measuring a peptide protease substrate which is cleaved in live cells. Conversely, we detected a decrease in viability in non-ST6Gal-I expressing counterpart cell lines indicating that cells lacking ST6Gal-I expression are more sensitive to cisplatin-mediated cell death. Finally, we observed that cisplatin-mediated cell death is attenuated by treating cells with blocking antibodies to Fas, consistent with other studies implicating this receptor in cisplatin efficacy. We postulate that the negatively-charged sialic acid addition to the Fas receptor interferes with receptor internalization after cisplatin treatment. Our findings suggest a novel mechanism contributing to intrinsic platinum drug resistance in ovarian cancer cell populations and identify ST6Gal-I as a promising target for therapeutic intervention.