COVER SHEET

TITLE: **Immune Cell-Ovarian Tumor Cell Adhesion through MUC16 and Immunocytokine**

AUTHOR'S NAME: **Sachi Horibata**

MAJOR: **Biochemistry**

DEPARTMENT: **College of Agricultural Life and Sciences**

MENTOR: **Manish Patankar**

DEPARTMENT: **Obstetrics and Gynecology**

YEAR: **2010**

ABSTRACT

IMMUNE CELL-OVARIAN TUMOR CELL ADHESION THROUGH MUC16 AND IMMUNOCYTOKINE

Natural killer (NK) cells are immune cell types that function normally to eliminate tumor cells by forming an immune synapse. However, we have shown that the presence of mucin, MUC16, on the surface of epithelial ovarian tumor cells inhibits the function of NK cells in two ways: 1) steric barrier 2) inducing inhibitory signaling through Siglec-9. To increase recognition of tumors by NK cells, we utilized the immunocytokine, KSIL-2, which activates NK cells through IL-2 and mediates ADCC via the KS antibody. Using a plate adhesion assay, we demonstrate that KSIL-2 mediates increased NK-tumor cell interactions. Our data shows that KSIL-2 can overcome the immune evasion mediated by the large MUC16 molecule.

SACHI HORIBATA / BIOCHEMISTRY
Author Name/Major

Author Signature

April 26, 2010
Date

MANISH PATANKAR / OB-GYN
Mentor Name/Department

M.S. Patankar. 4/28/2010
Mentor Signature

IMMUNE CELL-OVARIAN TUMOR CELL ADHESION THROUGH MUC16 AND IMMUNOCYTOKINE

Abstract

Natural killer (NK) cells are immune cell types that function normally to eliminate tumor cells by forming an immune synapse. However, we have shown that the presence of mucin, MUC16, on the surface of epithelial ovarian tumor cells inhibits the function of NK cells in two ways: 1) steric barrier 2) inducing inhibitory signaling through Siglec-9. To increase recognition of tumors by NK cells, we utilized the immunocytokine, KSIL-2, which activates NK cells through IL-2 and mediates Antibody-Dependent Cell Mediated Cytotoxicity (ADCC) via the KS antibody. Using a plate adhesion assay, we demonstrate that KSIL-2 mediates increased NK-tumor cell interactions. Our data shows that KSIL-2 can overcome the immune evasion mediated by the large MUC16 molecule.

Introduction

Ovarian Cancer: A Deadly Gynecologic Malignancy

Ovarian cancer is the fifth leading cause of cancer related deaths among women; yet, our understanding of the biology of this disease is limited [1]. In the United States alone, approximately 22,220 women are diagnosed and 16,210 women die from this disease each year [2]. Among ovarian cancers, epithelial ovarian cancer is the major type of ovarian cancer which is approximately eighty percent of the cases. Epithelial tumors can also be divided into histolytic types: serous, mucinous, endometrioid, and clear cell [3]. Due to its complexity, the clear cause of this disease is still unknown.

Structure of MUC 16

Epithelial ovarian cancer cells over express the mucin called MUC16 [4,5]. This mucin is popularly known as the cancer marker, CA125, and is been shown to be important in promoting the growth and metastasis of ovarian tumors. MUC16 is a very complex molecule with many carbohydrate chains extending from the main protein backbone and is approximately 2.5 to 5 million Dalton in molecular weight [6]. The structure of MUC16 consists of N-terminal domain, tandem repeat domain, SEA (Sea urchin, Enterokinase, Agrin) domain, transmembrane and cytoplasmic tail dotains as shown in Figure 1 [7]. The tandem repeat is composed of 156 amino acids repeats from 9 to 60 times which elongates the structure of the MUC16. The MUC16 molecule is also very heavily glycosylated with both serine and threonine-linked and asparagine-linked oligosaccharides, making it have anti-adhesive properties from their large negative charge [8].

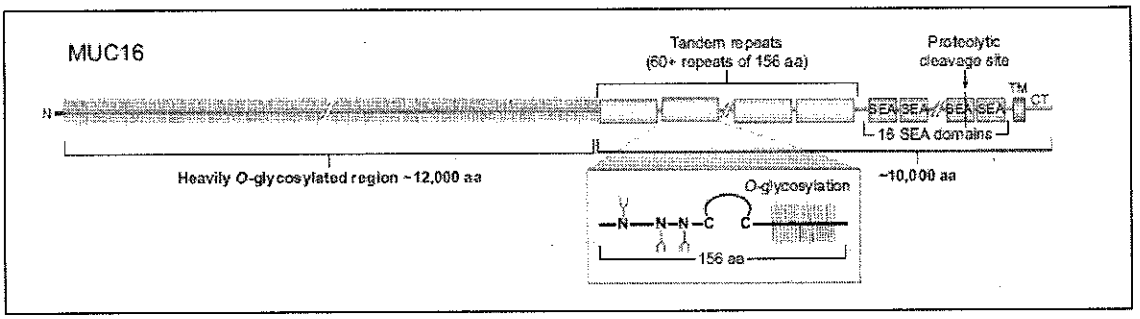


Figure 1: The Structure of MUC16. The figure represents the structure of MUC16. It is subdivided into N-terminal domain, tandem repeats domain, SEA (Sea urchin, Enterokinase, Agrin) domain, transmembrane, cytoplasmic tail domains and each tandem repeat is composed of 156 amino acids. Depending on the number of tandem repeats, the length of the mucin varies [7].

The shed MUC16 and cell surface MUC16

MUC16 is initially expressed on the surface of the tumor cells and can be detected in the peritoneal fluid and peripheral blood of the ovarian cancer patients. The shed MUC16 is an inhibitor of the cytolytic ability of natural killer (NK) cells and decreases the expression of CD16, an Fc receptor. [9, 10] Low CD16 level results to an impaired NK cells mediated Antibody-Dependent Cell Mediated Cytotoxicity (ADCC) [11-16]. Although shed MUC16 has been studied in its role to protect tumors from immune cells, little is known about the cell surface MUC16. The current study was therefore undertaken to identify the mechanism in which cell surface MUC16 binds to immune cells.

MUC16 Promotes Ovarian Tumor Progression by Inhibiting Immune Cells

Despite the presence of immune cells, ovarian tumor cells have developed mechanisms to prevent immune recognition and attack. Previous studies have shown that MUC16 facilitates tumor metastasis and inhibits immune cell mediated lysis of tumor targets [6,9,10]. One such example is a downregulation of CD3ζ, a signaling molecule, which results in impairment of the immune response [17]. It is hypothesized that the suppression of immune cells is the reason for tumor proliferation and thereby promoting tumor growth [6]. In order for the immune cells to cytolyse tumor cells, they need to form an immune synapse. Molecules of the immune cells that are required for cytolysis of the tumor targets are polarized at the immune synapse. This results in elimination of the tumor cells. Our data will show that cell surface MUC16 promotes efficient immune synapse formation between ovarian tumor cells and NK cells.

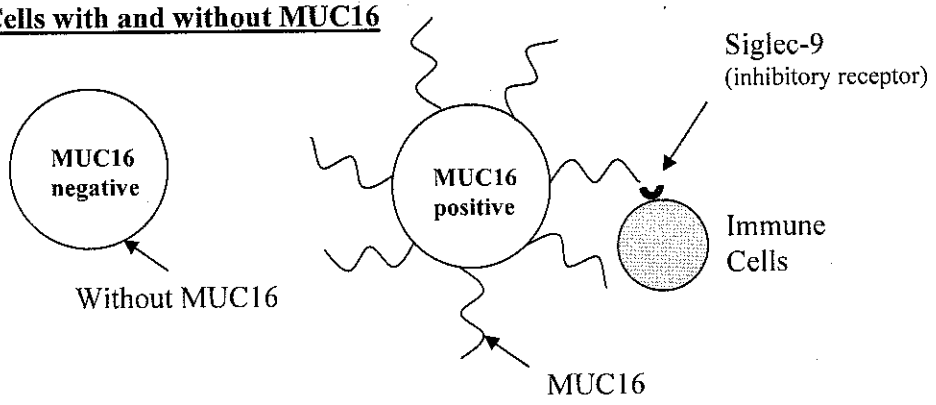
Immunocytokines and NK cells

NK cell is an immune cell type that functions normally to eliminate tumor cells from the body by interacting with the tumor cell and forming an immune synapse. NK cells are very highly activated by cytokines such as IL-2; therefore, molecules called immunocytokines are being tested to determine if they can increase the activity of NK cells in patients with cancer [18]. KSIL-2 is one such immunocytokine. KS is an antibody that is specific for a molecule on the tumor cell, and IL-2 is fused to one end of the antibody. Because of these properties, we hypothesize that this molecule will bring both the NK cell and the tumor cell into close contact by binding to both cell types. Here we test if the presence of MUC16 would adversely affect the biologic activity of KSIL-2.

Specific Aims

1. Determine if the presence of MUC16 can physically block the immune cells from forming an immune synapse with the tumor cells.
2. Determine whether MUC16 binds to the inhibitory receptor (Siglec-9) of the immune cells.
3. Investigate a possible immunotherapy to allow immune recognition and attack of tumor cells by the immune cells.

Diagram 1: The Simplified Structure of Tumor Cells with and without MUC16



The Goal of the Study

The goals of the study were to investigate whether MUC16 causes the metastasis of epithelial ovarian cancer due its abilities to block the immune cells from forming an immune synapse and from binding to the inhibitory receptor of NK cells, thus, inhibiting the function of the natural killer cells. Another goal was to determine the therapeutic potential of immunocytokines, KSIL-2, in treatment of ovarian cancer.

METHODS (Diagram 2)

To investigate the immunoregulatory role of MUC16, we used MUC16-positive-OVCAR cells (Ovarian cancer cells with MUC16) and MUC16-negative-OVCAR cells (Ovarian cancer cells without MUC16) that originated form the parent epithelial ovarian cancer cells. Based on data obtained in Dr. Patankar's lab, we particularly demonstrated that the immune receptor, Siglec-9 may serve as a binding partner of MUC16. To further investigate this molecular interaction, we conducted specific cell binding assays with monocytes and Siglec-7 and Siglec-9 expressing Jurkat cells (Diagram 1).

Specific Aim 1: Immune Synapse Experiment

MUC16-positive-OVCAR cells or MUC16-negative-cells were plated at 30,000 cells/well of a 96-well plate. These were incubated for 2 days to reach 100% confluency. Natural Killer Leukemia (NKL) cells were counted and dyed with 1uL calcein AM in 10mL of 1% PBS-BSA for 30 min in the water bath at 37°C. Different amounts of dyed NKL cells (25,000 cells, 50,000 cells, and 100,000 cells) were added to each well with MUC16-positive-OVCAR cells and MUC16-negative-cells. Each treatment was replicated 6 times. The plate was centrifuged to make the cells stick at the bottom of the plate. This plate was then incubated for 25 minutes. The plate was washed with 1% PBS-BSA 2 to 3 times to remove the cells that did not bind to the original cells in the plate which were either MUC16-positive-OVCAR cells or MUC16-negative-cells. The fluorescence emitted by the stained NKL cells remaining in the wells was measured using Perkin Elmer Victor 3-V plate reader.

Specific Aim 2: Receptor/Ligand Binding Experiment

Procedure similar to that used for specific aim 1 was employed to conduct the receptor/ligand binding experiments. Calcein labeled Siglec-7 and Siglec-9 expressing Jurkat cells were plated over the previously plated tumor cells. MUC16-positive-OVCAR cells, MUC16-negative-cells or OVCAR 3 (Ovarian Cancer Cell-line 3) were plated at 100,000 cells/well of a 96-well plate. These were incubated for 2 days to reach 100% confluency. Then, 100,000 of calcein labeled Siglec-7 and Siglec-9 expressing Jurkat cells were added and centrifuged. After 25 minutes of incubation, the non-adherent cells were removed by washing and the remaining cells were quantified using a fluorescence plate reader to determine whether Siglec-9 expressing Jurkat cells binds more efficiently with the ovarian tumor cells expressing MUC16.

Specific Aim 3: Immunocytokine Experiment

NKL cells were washed, counted, and dyed using 1uL calcein-AM in 10mL of PBS-BSA for 30 minutes at 37°C and at 5% CO2. The NKLs were then washed, separated into 6 tubes, and mixed with one of the following treatments: no treatment control, KSIL-2 (0.35uL), KS(1.25uL), IL-2 (4.80uL), or KS (1.25uL) + IL-2 (4.80uL), KSIL-2 (0.35uL) + TAC (40uL per 1mL of sample). Immediately after treatment, 100,000 NKLs cells from each treatment were added to 96-well plate with previously incubated OVCAR3 cells. The plate was placed in the incubator for 25 minutes, and then the NKLs were removed from the wells and this was followed by washing with PBS-BSA. The plate was immediately read on a Perkin Elmer Victor 3-V plate reader for calcein-AM concentration.

The Perkin Elmer Victor 3-V plate reader will provide quantitative data showing cell binding between immune cells and ovarian tumor cells. A binding is an indication that over a period of time, immune cells will lyse ovarian tumor cells. Thus, the higher number of cell binding or fluorescence level, the more tumor cell killing.

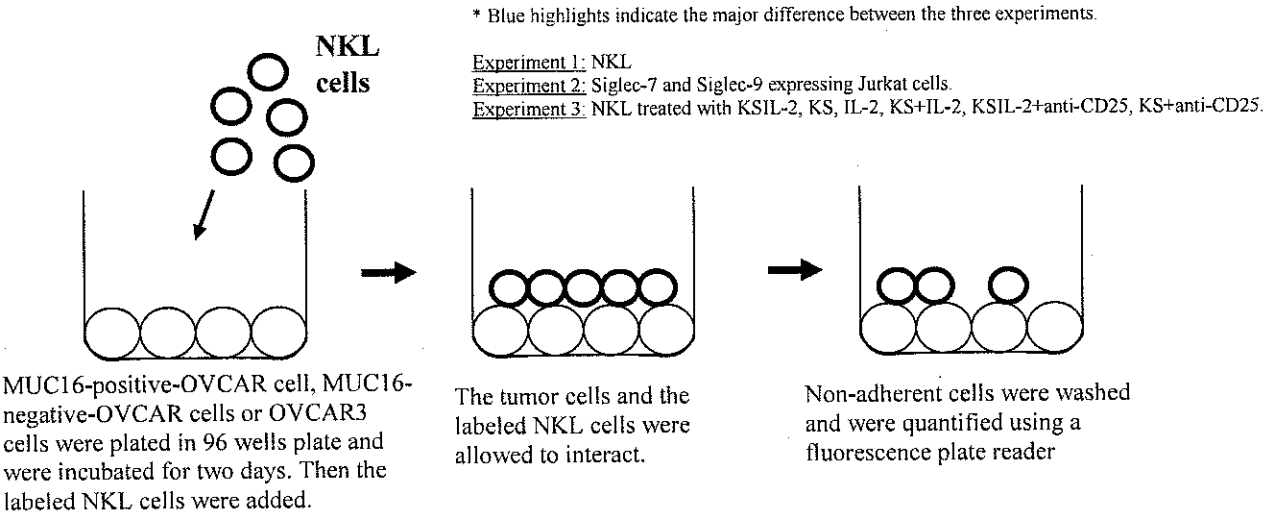


Diagram 2: Perkin Elmer Victor 3-V plate reader assay.

RESULTS

Experiment 1: ***Immune Synapse Experiment***

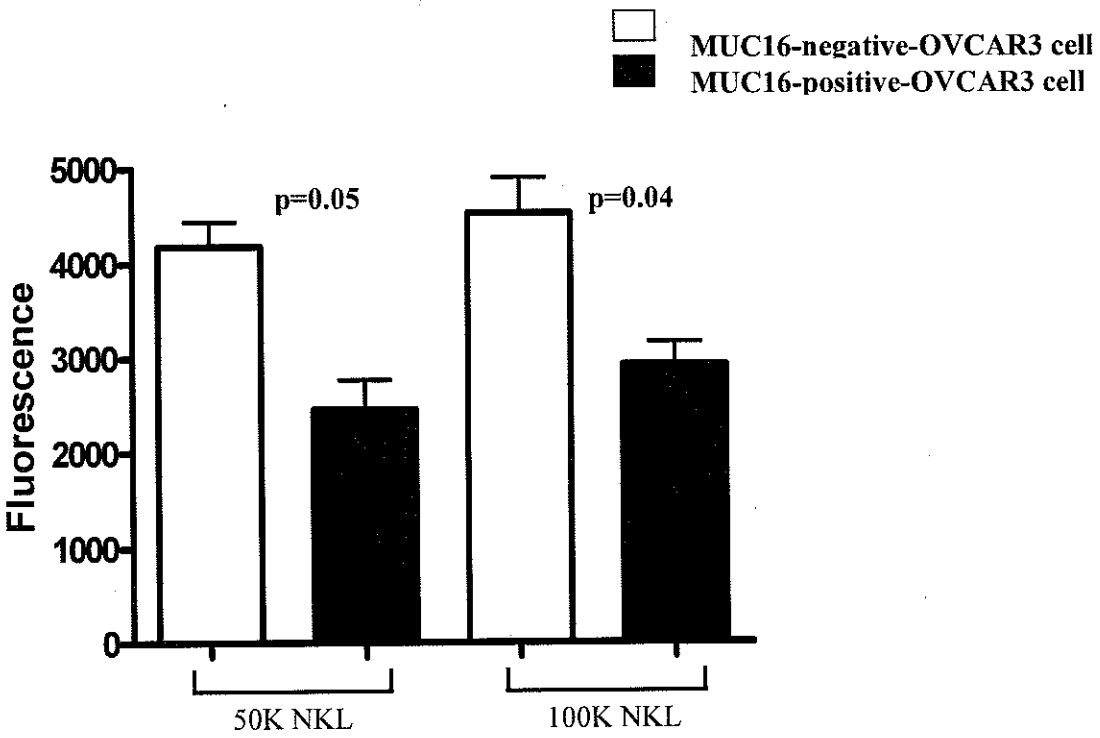


Figure 2: MUC16-negative-OVCAR3 cells form more conjugates with NKL cells than MUC16-positive-OVCAR3 cells. Cell surface MUC16-positive and MUC16-negative OVCAR3 cells were plated in a 96 well plate and were incubated to reach confluency. Calcein dyed NKL cells were added to the plate and were allowed to interact with the tumors. After 25 minutes, non-adherent cells were washed and the remaining bound NKL cells were quantified using a fluorescence plate reader.

12 repeated experiments were conducted for two experiments (Figure 2). Based on the observed data, cell surface MUC16-negative-OVCAR cells formed more binding with the NKL in plate adhesion assay than cell surface MUC16-positive OVCAR cells.

Experiment 2: ***Receptor/Ligand Binding Experiment***

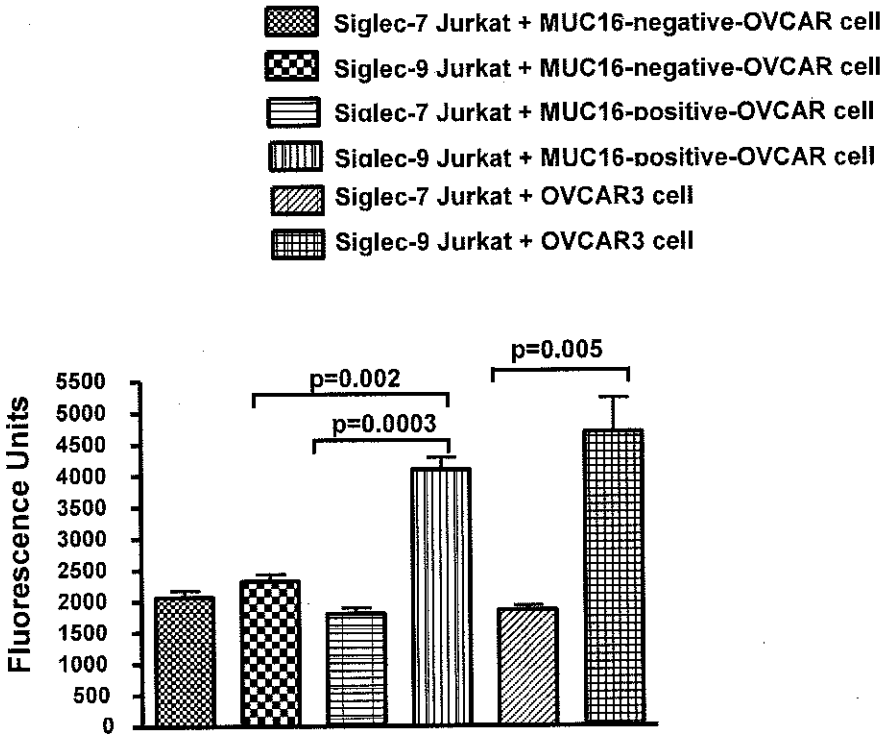


Figure 3: Siglec-9 binds to cell surface MUC16. Cell surface MUC16-positive and MUC16-negative OVCAR3 cells were plated in a 96 well plate and were incubated to reach confluency. Calcein dyed Siglec-7 and Siglec-9 expressing Jurkat cells were added to the plate and were allowed to interact with the tumors. After 25 minutes, non-adherent cells were washed and the remaining Jurkat cells were quantified using a fluorescence plate reader.

Six repeated experiments were conducted for each treatment (Figure 3). The binding between the MUC16-negative-OVCAR cells with either Siglec-7 or Siglec-9 expressing immune cells were almost similar. Also, binding between MUC16-positive-OVCAR cells or OVCAR3 cells with Siglec-7 expressing immune cells is similar. This is because both tumor cells express MUC16 and were treated with the negative control (Siglec-7 expressing immune cells). However, significantly more conjugates formation

was observed between Siglec-9 expressing Jurkat cells and cell surface MUC16-positive-OVCAR cells than between Siglec-7 expressing Jurkat cells and cell surface MUC16-positive OVCAR cells.

Experiment 3: ***Immunocytokine Experiment***

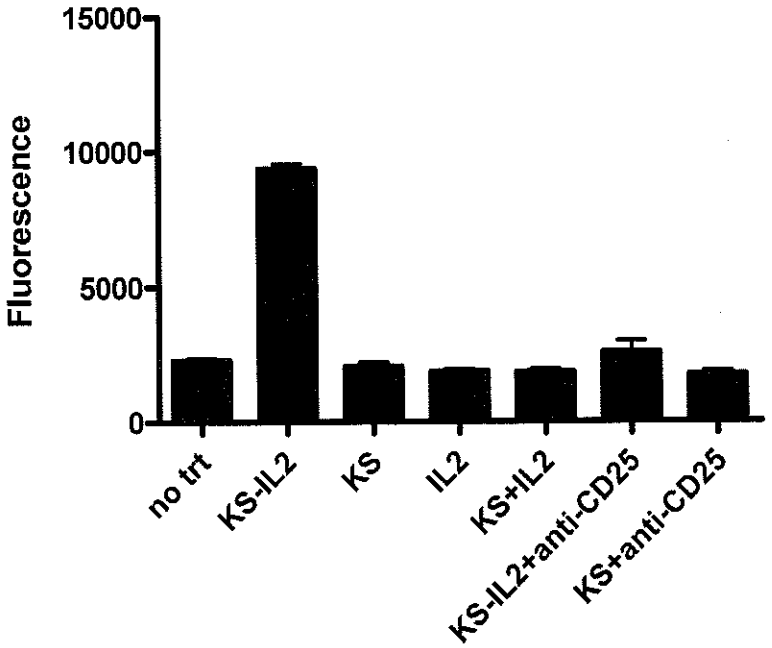


Figure 4: The presence of immunocytokine (KSIL-2) increased the binding between immune cells (NKL) and tumor cells. Cell surface OVCAR3 cells were plated in a 96 well plate and were incubated to reach confluency. Calcein dyed NKL cells treated with KSIL-2, KS, IL-2, KS+IL-2, KSIL-2+anti-CD25 and KS+anti-CD25were added to the plate and were allowed to interact with the tumors. After 25 minutes, non-adherent cells were washed and the remaining NKL cells were quantified using a fluorescence plate reader.

Six repeated experiments were conducted for each treatment (Figure 4). The binding between the NKL cells and OVCAR3 cells (express MUC16) was significantly

increased when treated with KSIL-2. However, OVCAR3 cells treatment with KSIL-2 in the presence of anti-CD25 decreased the number of binding to almost baseline.

DISCUSSION

Experiment 1: ***Immune Synapse Experiment***

HLA class I antigens signal to prevent cytotoxic ability of NK cells by serving as ligands for KIR (inhibitory) receptor. Since HLA class I level of cell surface MUC16-negative-OVCAR cells was lower than that of MUC16-positive-OVCAR cells (data not shown), it is expected that MUC16-negativce-OVCAR cells will be protected from the attack of NK cells. However, NK cells lysed more MUC16-negative-OVCAR cells than MUC16-positive-OVCAR cells. Our cell binding experiments and immune synapse assays demonstrate that MUC16 shields ovarian tumor cells from NK cell attack [19].

MUC16 exhibit both anti- and pro-adhesive properties. The data shows that the anti-adhesive property of MUC16 cirvumvent the immune synapse formation with the cytotoxic NK cells, thereby protect ovarian tumor cells from immune cell attack. In addition, MUC16 also promote cell metastasis through binding to mesothelial cells in the peritoneal cavity [6]. These collective properties of MUC16 likely result in increasing the tumor binding in ovarian cancer patients.

Experiment 2: ***Receptor/Ligand Binding Experiment***

The data has shown more binding between the Siglec-9 expressing Jurkat cells and cell surface MUC16-positive-OVCAR cells than MUC16-negatie-OVCAR cells. Although this means that Siglec-9 cells are in close enough contact to form immune synapse with the tumor cells, less killing of tumor cells observed. Previous studies have shown that the ligand binding results in the phosphorylation of tyrosine in Immunoreceptor Tyrosine-based Inhibition Motif (ITIM) of Siglec-9 and activation of phosphatases, SHP-1 and SHP-2 [20,21]. This activation of phosphatases causes inhibition of NK cell functions and secretion of immunosuppressive cytokine IL-10 [21].

Experiment 3: ***Immunocytokine Experiment***

Cytokine production occurs in the peritoneal cavity of the patients with ovarian cancer. Although cytokines contribute to the metastasis of tumor cells, it also activates NK cells to function as a defense against cancer [23]. When the ovarian cancer cells were exposed to NK cells with the presence of immunocytokines KSIL-2, there was more cell lysis. In other words, KSIL-2 activates NK cells through IL-2 and mediates ADCC via KS antibody. Activation of NK cells via KSIL-2 negates the protective effects provided by cell surface MUC16.

CONCLUSION:

From these studies, we have discovered that ovarian tumor cells protect themselves by physically blocking the immune cells and binding to the inhibitory receptor of the immune cells using MUC16. Siglec-9 was identified as the immune cell receptor for MUC16 and that protections of the tumor cells against immune cells were effectively lysed by the immunocytokines KSIL-2.

REFERENCE

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ: **Cancer statistics, 2007.** *CA Cancer J Clin* 2007, **57**:43-66.
2. DiSaia, P. J. (2007). *Clinical gynecologic oncology* (7th ed.). St. Louis, Mo.: Mosby Elsevier.
3. Schottenfeld, D., Fraumeni, J.F., & associateitors, Colditz, G.A., Samet, J.M., Whittemore, A.S. (2006). *Cancer epidemiology and prevention* (3rd ed.). Oxford; New York: Oxford University Press.
4. Kabawat SE, Bast RC, Jr., Bhan AK, Welch WR, Knapp RC, Colvin RB: **Tissue distribution of a coelomic-epithelium-related antigen recognized by the monoclonal antibody OC125.** *Int J Gynecol Pathol* 1983, **2**:275-285.
5. Niloff JM, Klug TL, Schaetzl E, Zurawski VR, Jr., Knapp RC, Jr.: **Elevation of serum CA125 in carcinomas of the fallopian tube, endometrium, and endocervix.** *Am J Obstet Gynecol* 1984, **148**:1057-1058.

6. Gubbels JA, Belisle J, Onda M, Rancourt C, Migneault M, Ho M, Bera TK, Connor JP, Sathyanarayana BK, Lee B, Pastan I, Patankar MS: **Mesothelin-MUC16 binding is a high affinity, N-glycan dependent interaction that facilitates peritoneal metastasis of ovarian tumors.** *Mol Cancer* 2006, **5**:50.

7. Hattrup CL, Gendler SJ: **Structure and funtion of the cell surface (tethered) mucins.** *Annu Rev Physiol* 2007, **70**:431-457.

8. Wong NK, Easton RL, Panico M, Sutton-Smith M, Morrison JC, Lattanzio FA, Morris HR, Clark GF, Dell A, Patankar MS: **Characterization of the oligosaccharides associated with the human ovarian tumor marker CA125.** *J Biol Chem* 2003, **278**:28619-28634.

9. Belisle JA, Gubbels JA, Raphael CA, Migneault M, Rancourt C, Connor JP, Patankar MS: **Peritoneal natural killer cells from epithelial ovarian cancer patients show an altered phenotype and bind to the tumour marker MUC16 (CA125).** *Immunology* 2007, 122:418-429.

10. Patankar MS, Yu J, Morrison JC, Belisle JA, Lattanzio FA, Deng Y, Wong NK, Morris HR, Dell A, Clark GF: **Potent suppression of natural killer cell response mediated by the ovarian tumor marker CA125.** *Gynecol Oncol* 2005, **99**:704-713.

11. Lai P, Rabinowich H, Crowley-Nowick PA, Bell MC, Mantovani G, Whiteside TL: **Alterations in expression and function of signal-transducing proteins in tumor-associated T and natural killer cells in patients with ovarian carcinoma.** *Clin Cancer Res* 1996, **2**:161-173.

12. Lotzova E, Savary CA, Freedman RS, Bowen JM: **Natural killer cell cytotoxic potential of patients with ovarian carcinoma and its modulation with virus-modified tumor cell extract.** *Cancer Immunol Immunother* 1984, **17**:124-129.

13. Connor JP, Felder M, Hank J, Harter J, Gan J, Gillies SD, Sondel P: **Ex vivo evaluation of anti-EpCAM immunocytokine huKS-IL2 in ovarian cancer.** *J Immunother* 2004, **27**:211-219.

14. Mantovani A, Allavena P, Sessa C, Bolis G, Mangioni C: **Natural killer activity of lymphoid cells isolated from human ascitic ovarian tumors.** *Int J Cancer* 1980, **25**:573-582.

15. Allavena P, Zanaboni F, Rossini S, Merendino A, Bonazzi C, Vassena L, Mangioni C, Mantovani A: **Lymphokine-activated killer activity of tumor-associated and peripheral blood lymphocytes isolated from patients with ascites ovarian tumors.** *J Natl Cancer Inst* 1986, **77**:863-868.

16. Melioli G, Ferrari I, Casartelli G, Ragni N: **Lymphocytes isolated from the peritoneal fluid of women with advanced ovarian carcinoma differ significantly from autologous peripheral blood lymphocytes.** *Gynecol Oncol* 1993, **48**:301-307.

17. Taylor DD, Gercel-Taylor C, Lyons KS, Stanson J, Whiteside TL: **T-cell apoptosis and suppression of T-cell receptor/CD3-zeta by Fas ligand-containing membrane vesicles shed from ovarian tumors.** *Clin Cancer Res* 2003, **9**:5113-5119.

18. Weil-Hillman G, Voss SD, Fisch P, Schell K, Hank JA, Sosman JA, Sugamura K, Sondel PM: **Natural killer cells activated by interleukin 2 treatment in vivo**

respond to interleukin 2 primarily through the p75 receptor and maintain the p55 (TAC) negative phenotype. *Clin Cancer Res* 1990, **9**:2683-2691.

19. Gubbels JA, Felder M, **Horibata S**, Belisle J, Holden H, Petrie S, Migneault M, Rancourt C, Connor J and Patankar MS: **MUC16 provides immune protection by inhibiting synapse formation between NK and ovarian tumor cells.** *Mol Cancer* 2010, **9**:11.
20. Ikehara Y, Ikehara SK, Paulson JC: **Negative regulation of T cell receptor signaling by Siglec-7 (p70/AIRM) and Siglec-9.** *J Biol Chem* 2004, **279**:43117-43125.
21. Avril T, Floyd H, Lopez F, Vivier E, Crocker PR: **The membrane-proximal immunoreceptor tyrosine-based inhibitory motif is critical for the inhibitory signaling mediated by Siglecs-7 and -9, CD33-related Siglecs expressed on human monocytes and NK cells.** *J Immunol* 2004, **173**:6841-6849.
22. Ando M, Tu W, Nishijima K, Iijima S: **Siglec-9 enhances IL-10 production in macrophages via tyrosine-based motifs.** *Biochem Biophys Res Commun* 2008, **369**:878-883.
23. Punnonen R, Teisala K, Kuoppala T, Bennett B, Punnonen J: **Cytokine production profiles in the peritoneal fluids of patients with malignant or benign gynecologic tumors.** *Cancer* 1998, **83**:788–796.