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The immunomodulating roles of glycoproteins in epithelial ovarian cancer

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1. ABSTRACT

The complexity of the immune system demands an intricate defense mechanism by tumors. Ovarian and other tumors employ specific glycoproteins and the associated glycan sequences to modulate immune responses. Glycoproteins enable tumor cells that express or secrete these molecules to evade immune cell attack and induce the immune system to promote tumor growth. This review focuses first on the immune environment in ovarian cancer, and the mechanisms of activation and inhibition that immune cells undergo in order to either attack or ignore a target cell. Next we illustrate the immunomodulatory roles of ovarian cancer-associated glycans and glycoproteins in 1. preventing immune synapse formation, 2. serving as ligands of immune cell receptors, 3. scavenging cytokines and chemokines, and 4. participating in the formation of autoantibodies against the tumor. The importance of these immunomodulating strategies from the view points of understanding the tumor immunology of ovarian tumors, potential origin of such mechanisms, and specific strategies to circumvent the glycoconjugate-mediated suppression of immune responses is discussed in this review.

2. INTRODUCTION

Ovarian cancer is a highly insidious disease that is usually detected at a very late stage when the possibility of an efficient therapeutic management of the cancer is relatively low (1-3). Therefore, in most women with advanced ovarian cancer the five year survival rate is around 30-55% (4). Specific biomarkers that can detect the cancer at an early stage (when the survival rate after treatment is 80%) are not available (5, 6). In women with advanced disease, the standard of care includes an initial surgical debulking of the tumor followed by an intense chemotherapy with platinum or taxol based compounds. Under these treatment conditions the cancer regresses and the low level of the tumor is monitored by measuring the serum concentration of the biomarker CA125 (7-10). A steady elevation in serum CA125 levels from this nadir is indicative of recurrent disease (10).

The progression of ovarian cancer requires the cancer cells to first develop on the surface of the ovary or along the walls of the fallopian tubes and then metastasize to other sites within the peritoneum (11-18). Starting with

the initiation of the cancer to metastasis, the tumor cells encounter distinct immunologic environments and therefore have to adapt at each site to not only overcome immune recognition but also actively suppress cytotoxic immune responses. Modulation of the immune responses is achieved via various strategies, including downregulation of MHC Class I molecules, expression of soluble MICA, MICB and other ligands of the immune activating receptor NKG2D (19, 20-23), expression of immunosuppressive cytokines, induction of regulatory T cells (24-27), and others. Another important mechanism displayed by ovarian (and other tumors) involves the selective expression of immunemodulating glycoconjugates. In this review, we will discuss the biological properties of well characterized glycoconjugates expressed by ovarian tumors and their effects on immune cells that likely lead to the generation of a diverse array of redundant mechanisms that allow protection of ovarian cancer cells from immune attack. While a major emphasis will be on the discussion of the effects of specific glycoproteins on cellular immunity, we will also briefly discuss auto-antibody responses against glycoproteins and the disease-specific changes in glycosylation occurring on IgGs that may reduce their ability to trigger humoral immune responses via the Fc receptors. An overview of the literature on tumor immune surveillance and the immune environment associated with ovarian tumors is initially provided to set the stage for the discussion of the immunological relationships with the glycoproteins expressed by ovarian tumors.

3. IMMUNE SURVEILLANCE AND IMMUNE EDITING

Early reports of active immune surveillance have now been validated by several investigations and explained by the immunoediting model described by Robert Schrieber and colleagues (28, 29). According to this model, the immune system is continuously encountering and eliminating aberrant tumorigenic cells (immune surveillance) (30-34). While successful elimination occurs in the majority of the cases, in some instances the immune system is unable to completely cytolyse the aberrant lesions. In such cases, a stasis is reached where the immune cells co-exist with the aberrant lesion. It is likely that at this stage the immune system is also undertaking immune surveillance and eliminating some aberrant cells but is unable to completely eliminate the lesion. On the other hand, the aberrant cells that are not being eliminated have likely developed mechanisms that allow them to escape immune recognition. Certain genetic and other molecular events trigger these pre-cancer lesions to break through the equilibrium with the immune cells as they become highly malignant while retaining or adding to the mechanisms of immune evasion and immune suppression that they have already developed during the equilibrium stage (30, 35, 36). The exact nature of these immune evasion or immune suppression strategies are currently under intense investigation and are likely to be cancer-specific. The discussion provided in this review will indicate that specific glycoproteins likely mediate an immunomodulating role during the entire lifecycle of the tumor that enables the cancer cells to escape immune recognition in diverse immune environments.

4. IMMUNE ENVIRONMENT IN OVARIAN CANCER

Ovarian tumors are primarily confined to the peritoneum. Cancer cells sloughing off from the primary tumor metastasize to the diaphragm, oomentum and other sites within the peritoneal cavity. Advanced stages of the cancer are associated with significant accumulation of ascites (37, 38). As a part of standard care to relieve discomfort and bloating, this peritoneal exudate is surgically extracted from patients. An analysis of the total cellular component of the ascites indicates approximately 1-5% of the cells are tumor cells and 40-60% are likely of stromal or mesothelial origin (Felder, Belisle and Patankar unpublished observations). The remaining proportion of the cells are predominantly $CD45^{pos}$, indicating that they are of immune origin. These peritoneal immune cells are continuously exposed to tumor antigens that affect their phenotype and function (24, 39-41). Thus ovarian cancer affords an opportunity to simultaneously analyze immune cells in peripheral circulation that are relatively less affected by the tumor, the peritoneal immune cells that are in direct contact with tumor derived factors, and finally also the immune cells that infiltrate into the ovarian tumor mass (25, 41-45).

The effect of immune response on patient survival has been extensively studied in ovarian cancer. The infiltration of $CD4^{pos}$ cytotoxic T cells is associated with increased survival whereas negative correlation with disease free survival is observed in patients with increased numbers of regulatory T cells (T_{reg}) within the tumor (25, 44, 45). Lymphocytes in the peritoneal fluid of ovarian cancer patients include T cells, B cells, NK cells and monocytes in roughly the same proportion as those found in the peripheral circulation of the patients (42, 46). However, major phenotypic differences, especially in the NK cells, are observed among the peritoneal cells as compared to the peripheral blood derived immune cells (42). A higher percentage (60-70%) of the peritoneal NK cells express low levels of the Fc receptor CD16 and higher levels of CD56, a phenotype strongly associated with low cytolytic function (47-49). Our subsequent discussions will show that such changes in immune cell phenotype and the associated differences in immune function are likely due to a contribution from ovarian cancer associated glycoconjugates.

5. ANTI-TUMOR IMMUNE RESPONSE

Immune reaction against tumors may occur via humoral responses as well as cellular cytotoxicity. In subsequent discussion, we will review data on the generation of autoantibodies, especially against glycoprotein antigens, in ovarian cancer patients. Cellular cytotoxic responses occur via the activation of the adaptive as well as the innate immune system. Typically the cytotoxic T cells (adaptive immune response) and NK cells (innate immune response) are prominent mediators of anti-cancer cytotoxicity. The decision by the T cells and NK cells to kill the target cells or not is made depending on the interaction of the activating or the inhibitory receptors of

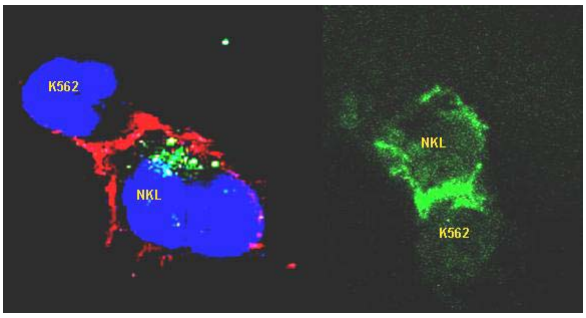

Figure 1. NK immune synapse. Localization of actin (red) at the contact site between NKL (an NK leukemia cell line) and K562 (target) cells is shown in the left panel. Nucleus stained with DAPI (blue) and perforin (green) is in transition towards the synapse. Recruitment of LFA (green) to the contact site is shown in the right panel.

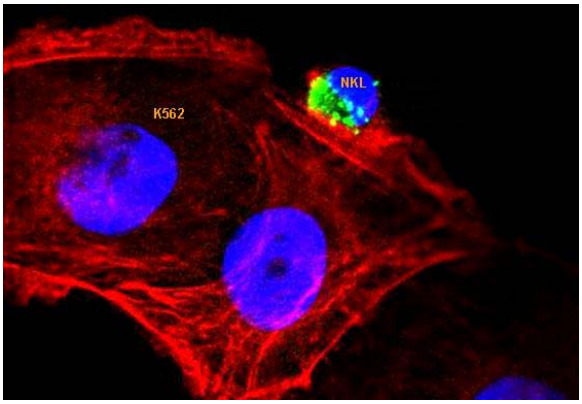

Figure 2. Recruitment of perforin granules to the NK immune synapse. Synapse between K562 cells (labeled with phalloidin to stain actin, red) and NKL (NK cell leukemia cell line) is shown. Perforin granules (green) are recruited to the synapse. Nucleus is stained with DAPI (blue).

the immune cells with their corresponding ligands on the tumor targets via the formation of an immune synapse (Figure 1) (50-52). Thus, an increase in expression and recognition of inhibitory ligands on the tumor cells by the immune cells results in inhibition of the cytotoxic immune response. This ultimately leads to survival of the targets. This inhibitory response is essential for the normal regulation of NK and cytotoxic T cells to prevent them from attacking normal self cells. On the other hand, high expression of activating ligands on the target cells results in the engagement of activating receptors of the immune cells (53). Specific cell signaling events triggered through these interactions between the activating receptors and their ligands result in the lysis of the tumor cells via recruitment of perforin and granzymes to the immune synapse (Figure 2).

One major class of inhibitory immune receptors includes the Killer Immunoglobulin-like Receptors (KIR) (54-58). Prominent examples of the KIR family of receptors expressed on NK cells and T cells include the KIR2DL1, KIR2DL2, KIR2DL3, KIR3DL1. Specific allotypes of MHC class I molecules are ligands of the KIRs. KIR2DL1 recognizes HLA-C2, KIR2DL2 and KIR2DL3 recognize HLA-C1, and KIR3DL1 recognize HLA-Bw4. Cancer cells expressing these MHC class I molecules are protected from cytolysis by NK or T cell clones that express the corresponding KIRs.

Other inhibitory immune cell receptors that are important in our discussion of tumor-immune interactions include CD94/NKG2A (recognizes the leader sequence of HLA-E as ligand) (59, 60), LIR-1 (Leukocyte Immunoglobulin-like Receptor-1; recognize HLA-G as ligands) and other LIRs (61, 62), Siglecs (I-type lectins that recognize terminal sialic acids residues) (63-66). Engagement of the inhibitory receptors mediates strong attenuation of the immune response via recruitment of the phosphatases SHP-1, SHP-2 and SHIP (67-70).

Several activating receptors are also expressed by cytotoxic immune cells and their engagement is vitally important for cell mediated cytotoxicity against tumor cells. Fc receptors constitute a potent activating receptor system that binds to antigen bound antibodies and causes a high level of activation of the cytotoxic cellular immune response. Other activating receptors include NKG2D, DNAM-1, NKp30, NKp44, NKp46, and others. From the perspective of ovarian cancer, the NKG2D and DNAM-1 receptor systems are of importance because ovarian tumors express ligands for these two receptors (23, 71-75). It has been proposed that naïve or activated autologous NK cells can mediate cytolysis of ovarian tumors because of the expression of NKG2D and DNAM-1 ligands by ovarian tumors (76). However, it should be noted that the killing of ovarian tumor cells by NK cells is significantly lower than the killing observed with NK cell susceptible targets (Figure 3). This is likely because ovarian tumor cells have developed elaborate mechanisms to circumvent NK cell killing.

6. THE DECISION TO KILL

The expression of the array of inhibitory and activating receptors implies that the decision by the T cells and NK cells to kill their targets is made via a complex series of molecular events (51, 77). This decision is the net result of the interactions of the inhibitory and the activating receptors with their corresponding ligands on the target cells. The strength (and number) of the receptor-ligand interaction appears to be a critical factor in deciding whether the immune cells will be inhibited or activated. Inhibitory KIRs generate strong negative responses. Indeed blocking of the KIR-MHC interactions in ovarian cancer cells (Felder, Gubbels and Patankar, unpublished observations) results in increased cytolysis of ovarian tumor cells by naïve healthy donor derived NK cells. The strong inhibition of the cytotoxic responses via the KIRs likely ensures a non-response to MHC class I antigen expressing normal self cells.

From the perspective of the tumor, an over reliance of the MHC class I-KIR inhibition may result in

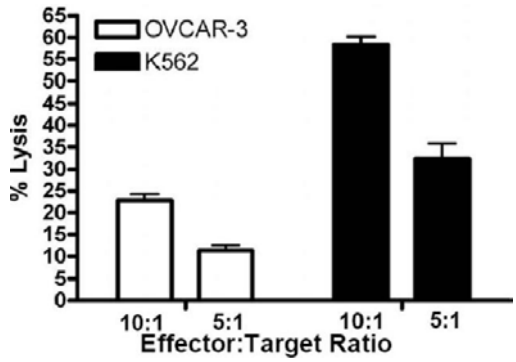

Figure 3. Reduced susceptibility of ovarian tumor cells to NK cell mediated lysis. Ability of NK cells derived from healthy donor peripheral blood to lyse the ovarian tumor cell line OVCAR-3 in comparison to the NK cell susceptible cell line, K562, is shown. Cytotoxicity was determined in a 4 h chromium release assay.

presentation of aberrant peptide antigens on its MHC molecules that can be identified by the T cell receptor (TCR) system leading to a strong adaptive response. Thus the tumor cells cannot rely completely on the KIR-MHC recognition for protection from the immune response. Redundant mechanisms must be displayed by the tumor that collectively results in immune protection. It is in this context that we should consider the potential immunomodulatory properties of specific glycan-based strategies displayed by ovarian and other cancers to protect themselves from immune attack.

Another important point to consider is that the tumor is displaying such redundant mechanisms not only to evade the immune response but also to mediate immune suppression and to co-opt the immune cells to produce trophic factors that promote tumor angiogenesis and hence tumor growth. In other words, the effect from the immune system's perspective is akin to the "Stockholm syndrome" experienced by kidnapping victims who through severe psychological manipulation are made to help their kidnapper.

7. GLYCOPROTEINS AND IMMUNE PROTECTION

Glycoconjugates expressed by cancer cells play important roles in promoting metastasis and angiogenesis. For example, binding of the ovarian tumor mucin MUC16 to mesothelin, a binding interaction that likely occurs via the N-glycans of MUC16, allows the cancer cells to attach to themselves to form spheroids and also for the cancer cells to metastasize within the peritoneal cavity (78-81). Heparan sulfate proteoglycans expressed by tumors regulate angiogenesis by serving as co-receptors for VEGF and FGF (82-85) Thus; glycoconjugates play important roles during the progression of ovarian and other cancers. In this review we will discuss the immunomodulatory properties of glycoproteins (especially of the glycoproteins expressed by ovarian cancer cells) that allow the tumor cells to circumvent immune responses throughout the life cycle of the cancer.

While considering the immunomodulatory effects of glycoproteins in ovarian and other tumors, it is important to consider the biology occurring at the surface of tumor cells as well as in the extracellular milieu around the tumor cells and the immune cells. This is because immunomodulation can arise through the glycoproteins that are expressed on the surface as well as those secreted by the tumor cells.

Glycoproteins can modulate immune activity via at least four prominent mechanisms- (i) providing a glycoprotein shield around tumor cells that prevents the immune cells from forming immune synapses; (ii) interaction with immune cell receptors; (iii) serve as scavengers of cytokines or chemokines; and (iv) generation of autoantibodies against aberrantly glycosylated self-glycoproteins. We will now discuss specific examples to illustrate these four operational mechanisms by which gycoproteins are known to modulate immune responses against ovarian tumors.

It is important to mention that another mechanism used by tumor cells to induce apoptosis of T cells is not directly related to expression of glycoproteins but instead via the expression of galectins that can induce T cell death (86). Expression of galectin-1 is associated with aggressiveness of tumor cells (87-89). Galectin-1 and galectin-3 are expressed by ovarian tumors (90-92). Significant data is now available indicating that galectin-1 binding to N-linked and O-linked glycans of CD45 and other molecules expressed on T cells induces immune cell apoptosis (93). Galectin-3 also regulates T cell apoptosis by controlling the aggregation of CD3 and CD8 on T cells (94). The expression of galectin-1 and galectin-3 by ovarian tumors may allow direct modulation of the function and viability of T cells and other types of immune cells thereby allowing the cancer cells to attenuate anti-tumor immune response.

7.1. Glycoprotein shield

Both the T cells and the NK cells require close cellular interactions with the cancer cells (Figure 1 and 2). These interactions are characterized by the polarization of cell adhesion molecules (LFA-1, ICAM-1), immune activating receptors, and cell signaling molecules at the contact sites between the tumor cells and the immune cells (53, 95-98). Such contact sites are referred to as the immune synapse. There are two types of immune synapses- inhibitory and activating. The inhibitory synapses occur through the engagement of inhibitory immune cell receptors with their corresponding ligands on the target cells. Similarly, activating immune synapses are a result of binding of immune activating receptors with their ligands. These inhibitory synapses result in the delivery of perforin and granzyme to the target cell resulting in cytotoxicity (Figure 2).

Since activating immune synapse formation is essential for the lysis of targets, prevention of such interactions leads to immune protection of the cancer cells. There is one specific strategy that ovarian tumors use to reduce the formation of activating immune synapses with

NK cells. This strategy involves the expression of high molecular weight mucins. Epithelial ovarian tumors over-express the mucins MUC1, MUC4 and MUC16 (99-106). Mucins are large molecular weight glycoproteins that are heavily glycosylated with both O-linked as well as N-linked oligosaccharides that add to the bulk of the molecule (100, 107). Structural studies have shown that the mucins display a linear extended structure. A glycopeptide consisting of 28 amino acids is estimated to extend up to 7 nm in length (108). Based on this estimate, MUC16 which is composed of ~22,000 amino acids is expected to extend between 1-5 micrometer on the surface of the tumor cells (109).

Because of this extended structure, the normal expression of mucins is known to provide a shield on the surface of epithelial cells. For example, the shielding effect of MUC16 prevents adhesion of Staphyloccocus aureus to corneal epithelial cells (110, 111). Similarly, expression of MUC1 and MUC4 on endometrial epithelial cells prevents the implantation of the embryo (112-117). Shedding of mucins, an event regulated by estradiol, TGF-beta and other mediators, leads to development of sites where the embryo can attach to the endometrial wall (116). In all of these studies it appears that because of their extended structure, mucins have the ability to introduce steric hindrance by exhibiting anti-adhesive effects that prevent cell-cell interactions.

A similar effect is also observed on the interaction between immune cells and cancer cells. Studies by Carraway and colleagues showed that the presence of MUC4 protected target cells from cytolysis by Lymphokine Activated Killer (LAK) cells (118). Recombinant fragments of MUC4 containing between 1-8 tandem repeats were tested in these experiments. It was demonstrated that the inhibitory effect of MUC4 was directly proportional to the number of tandem repeats within MUC4, as the constructs with 8 tandem repeats provided maximum protection (118). Although such a protective effect for MUC4 on ovarian cancer cells has not been demonstrated, recent data obtained in our laboratory on MUC16 gives credence to the importance of the shielding effect in protecting ovarian tumor cells from NK cell attack (109).

In co-cultures of the MUC16 expressing ovarian tumor cell line OVCAR-3 and human NK cells, it was invariably observed that the immune cells preferably formed conjugates with OVCAR-3 cells that expressed lower levels of the mucin (109). This initial observation led us to investigate the potential shielding effect of MUC16. Indeed, MUC16-knockdown OVCAR-3 cells formed increased numbers of immune synapses with the NK cells as compared to $MUC16^{pos}$ OVCAR-3 subclones. This increase in synapse formation was correlated with increased lysis of the MUC16-knockdown OVCAR-3 cells. The selective lysis of the MUC16-knockdown was not a result of increased expression of activating receptor ligands or conversely due to decreased expression of the ligands of inhibitory receptors on the MUC16-knockdown target cells. Similarly, no major difference was observed in the expression level of ICAM-1 between the MUC16 expressing and the MUC16-knockdown cells. All of these experiments indicated that the expression of MUC16 provided a steric barrier around the ovarian cancer cells resulting in the protection of the cancer cells from NK cell-mediated attack.

7.2. Immunemodulation via interaction of immune receptors with carbohydrate ligands

7.2.1. Ovarian tumor glycome

Glycoproteomic analysis of ovarian cancer cells and serum from ovarian cancer patients has resulted in the identification of aberrant glycosylation patterns of glycoproteins (119-121). Such aberrant glycosylation patterns may serve as diagnostic markers for ovarian malignancy (119-122). While a glycome-based diagnostic test may prove useful in early detection or monitoring of ovarian cancer, it will also be of great importance if the presence of a specific aberrant glycosylation pattern could predict poor survival of patients. To the best of our knowledge, such an analysis has not been conducted, although it would likely suggest that the biology elicited by specific glycans may contribute to the malignant potential of ovarian tumors. Initial analysis has not detected any changes in the expression patterns of $Lewis^y$ and Sialyl $Lewis^x$ and the tumor associated Sialyl Tn and Tn antigen levels on tumors from long term or short term survivors (123). However, in this study a trend of higher expression of $Lewis^y$ on tumors from short term survivors was observed (123). Several studies have indicated the expression of specific oligosaccharide sequences on ovarian tumor cells that have been previously shown to alter anti-tumor immune responses. We will review prominent oligosaccharide ligands expressed on the cell surface as well as secreted glycoproteins expressed by ovarian tumor cells that have been shown to possess the potential to attenuate immune responses.

7.2.2. Tn and Sialyl Tn antigens

The expression of Tn and sialyl Tn antigens have been observed on ovarian tumors and studies have also attempted to determine if monitoring the levels of these carbohydrate antigens in coordination with serum CA125 levels could be used to detect and monitor ovarian cancer progression (123, 124). Tn and sialyl Tn antigens have been detected on the O-linked glycans expressed on the ovarian tumor cell surface and these glycans have also been detected on MUC16 and other specific glycoproteins isolated from ovarian tumor cells (125).

The abundance of the Tn antigen in tumor associated mucins is linked with mutations in the molecular chaperone Cosmc (126-129). The Tn antigen (GalNAc alpha-Ser/Thr) is predominantly modified by the core-beta1-3Galactosyltransferase (T synthase) to the T antigen in normal cells (130). Mutations in Cosmc result in a deficit in the proper functioning of the T synthase, leading to increased expression of the Tn antigen in cancer cells (127, 128). The Tn antigen is further modified by sialylation in tumor cells to the sialyl Tn antigen (NeuAc alpha6-GalNAc alpha-Ser/Thr). Although mutations in Cosmc have been

detected in cervical tumors, a correlation has not been made in ovarian cancer (127). From an immunologic standpoint, the expression of Tn and sialyl Tn antigen is an important factor as these antigens possess the ability to inhibit the cytolytic responses of NK cells (131, 132).

The exact mechanism by which the Tn or sialyl Tn antigens inhibit NK cell responses are not clear. However, specific receptors for these antigens on immune cells exist. As we will discuss later, the Tn antigen may serve as a ligand for I-type lectins such as the Siglecs that are known to attenuate immune responses.

On the other hand, the Tn antigen is a ligand of the Macrophage galactose-type lectin (MGL, CD301), a C-type lectin expressed on immature dendritic cells (133, 134). This receptor was first identified on tumoricidal macrophages and exists as one single isoform in humans but as two separate isoforms, MGL-1 and MGL2, in mice (135-137). MGL binds to terminal GalNAc residues such as those presented by the Tn antigen (134). Binding of MGL to its ligands leads to internalization of the ligand-receptor complex (138). The internalized complex is transported to the endosomal compartments that contain MHC class II molecules. Hence, it has been postulated that MGL may play a role in cross presentation of the glycoprotein/glycopeptides ligands to the MHC class II molecules, thereby facilitating downstream adaptive response through the T cells.

MUC1 glycopeptides containing up to 3 tandem repeats of the mucin and engineered to express 9-15 mol of Tn antigen are internalized by dendritic cells through the binding with MGL (139). These glycocopeptides are delivered to endosomes containing both MHC class I and Class II molecules. However, these same studies have demonstrated that Tn antigen expressing MUC1 glycoprotein, although internalized by the dendritic cells through MGL was presented to endosomal compartments containing only the MHC class II and not the MHC class I antigens.

The presentation of MUC1 glycopeptides to endosomal compartments containing MHC class I molecules is an important finding from the perspective of developing anti-tumor vaccine strategies. Dendritic cells loaded with MUC1 peptides or glycopeptides are being investigated as a route to induce anti-tumor immunity (140, 141). Thus the interaction of the glycopeptides with MGL and their subsequent cross-presentation to the MHC class I molecules may indeed serve as an important route to develop novel anti-cancer dendritic vaccine strategies.

While internalization of MGL-ligand complex has been demonstrated in several studies, only recent studies have provided direct evidence for the development of T cell responses against the internalized antigens. GalNAc or MUC1 peptides expressing GalNAc when conjugated with streptavidin were internalized by dendritic cells (142). Such internalization was inhibited in $MGL2^{-/-}$ mice indicating that MGL2 was the primary receptor for these glycoconjugates. Incubation of the GalNAc-Streptavidin or the GalNAc-expressing MUC1-streptavidin complexes with dendritic cells resulted in high proliferation of CD4 T cells and increased ability of these T cells to produce IFN-γ and IL-17 by streptavidin primed CD4 T cells (142). Thus, MGL binding to its glycan ligands can result in presentation of peptide epitopes of glycoproteins on MHC class I molecules.

Presentation of the Tn antigen to MGL expressing dermal dendritic cells also results in internalization of the complex and delivery of the glycans to MHC class II (143). The net result is development of T and B cell responses against the Tn antigen, further suggesting that the use of Tn based vaccination strategies may play an important role in controlling tumor growth (143).

7.2.3. Thomsen-Friedenrich (T or Tf) antigen

The Tn antigen was first described by Dausset et al. in a patient with hemolytic anemia (144). Prior to this observation, another antigen described as the T antigen was also identified by Thomsen and Friedenreich (145). The T antigen (Galbeta1-3GalNAc), also known as the Thomsen-Friedenreich or Tf antigen, is exposed on red blood cells infected with viruses and bacteria or after digestion with neuramindase (146). Pioneering research conducted by George Springer led to the understanding that the majority of solid tumors express high levels of T antigen on their surface (147, 148). Springer and colleagues further demonstrated that the majority of adults have circulating IgM autoantibodies against T antigen (149). A quotient developed by this group (Q_{Me}; calculated as the square of the anti-T antigen IgM concentration divided by the concentration of the total IgM times 100) led to the observation of significantly lower levels in patients with breast, colorectal, gastric and many other carcinomas (148, 150). Interestingly, the Q_{Me} provided high specificity to not only differentiate between healthy individuals and cancer patients but also between benign conditions and malignancies (148, 150). The passing of Dr. Springer halted the development of the Q_{Me} quotient as a pan-cancer diagnostic tool for early detection of ovarian and other tumors and a clinical trial involving the repeated administration of a T/Tn antigen vaccine to breast cancer patients. Data available from this trial indicated that 94% (17/18) and 100% (18/18) of the patients with stage III and IV breast cancer, respectively, survived for five years after initiation of treatment (151, 152). This impressive survival advantage led others to develop T and Tn antigen-based vaccination strategies that involved the activation of the immune response by the Keyhole Limpet Hemocyanin and other adjuvants or by using synthetic constructs of T and Tn antigens (153, 154). Clinical trials are currently underway to determine the efficacy of these T and Tn antigen based vaccination approaches. The inability of carbohydrate antigens to produce T cell responses is likely the cause of the lack of a robust clinical response in these T/Tn antigen based vaccination approaches (155).

7.2.4. LacDiNAc glycans

Glycomic analysis of the ovarian cancer cell line SKOV-3 has indicated the expression of LacdiNAc

(GalNAcbeta1-4GlcNAc-) sequence on surface glycoproteins (156). Recombinant erythropoietin expressed by SKOV-3 cells also expresses the LacdiNAc sequence. The very first reports of LacdiNAc expression was on the surface of helminthic parasites (157, 158). Glycomic profiling of the pregnancy associated human glycoprotein, glycodelin-A, showed for the first time the presence of LacdiNAc terminated N-linked oligosaccharides on this glycoprotein (159). Early studies had shown that glycodelin-A (also referred to previously as PP14 and PAEP (159-161)) was a potent inhibitor of human NK cell mediated cytotoxic responses against the erythroleukemia cell line K562 (162). Although the exact mechanism by which glycodelin inhibits NK cell responses has not been elucidated, the LacdiNAc sequences are known to be ligands of galectin-3, a galactose binding lectin that is expressed on regulatory T cells and is known to induce apoptosis of T cells, and regulate T cell receptor-mediated activation of T cells (163-167).

7.2.5. Bisecting-type N-linked glycans

The enzyme N-acetylglucosaminyltransferase III, encoded by the Mgat3 gene, is responsible for the addition of the bisecting beta1-4-linked GlcNAc residues to complex type N-glycans (168). Deletion of the Mgat3 gene is associated with reduced development of mammary, liver, and other tumors (169, 170). Mass spectrometric analysis of MUC16 indicated abundant expression of the bisecting glycans on this mucin (125). Two crucial studies had previously implicated the bisecting type N-linked oligosaccharides in protecting tumor cells from NK cell-mediated lysis (171, 172). These initial reports originally inspired us to investigate the immunosuppressive activities of MUC16 (173). However, while the bisecting glycans are expressed not only on cancer associated glycoproteins but also on glycoproteins expressed by HIV (174, 175) and other pathogens, a clear immune receptor through which these oligosaccharide chains may develop an immunosuppressive response has not emerged.

7.2.6. Sialylated N-linked or O-linked glycans

Sialylation of glycolipids and glycoproteins affects the recognition of tumor cells by immune cells (176-181). It is generally accepted that a decrease in sialylation of cell surface glycans results in increased recognition of the cancer cells by immune cells. The data is particularly striking for NK cell recognition of tumor cells. The ovarian tumor mucin MUC16 is a potent inhibitor of NK cell mediated cytolytic response (173). We have recently demonstrated that MUC16 is a high affinity ligand of the NK cell receptor Siglec-9 (182). An I-type lectin, Siglec-9 specifically recognizes alpha2-3-linked sialic acid residues (183, 184) that are abundant on MUC16 (125). Siglec-9 is primarily expressed on the surface of innate immune cells (182). High levels of Siglec-9 are expressed on neutrophils and monocytes whereas intermediate levels of this receptor are present on approximately 30-40% of $CD16^{pos}/CD56^{dim}$ NK cells and approximately 5-10% of $CD19^{pos}$ B cells (182, 185). Low levels of Siglec-9 are present on 1-5% of $CD3^{pos}$ T cells (186).

In our studies on understanding the mechanism by which MUC16 inhibits NK cell cytolytic responses, we observed that NK cells and monocytes from the peripheral blood and peritoneal fluid of ovarian cancer patients were positive for this mucin (42, 182). RT-PCR indicated that the immune cells from the patients were not expressing endogenous MUC16. Instead, it was demonstrated that immune cells were binding to MUC16 released by ovarian tumor cells (42). Additional experiments led us to conclude that MUC16 was binding via its terminal sialic acid residues to Siglec-9 on the immune cells (182).

Siglec-9 is an inhibitory immune cell receptor. Engagement of Siglec-9 results in attenuation of T cell and NK cell cytolytic responses and data originating from monocytic cell lines suggests that signaling through this I-type lectin may induce monocytes to express the immunosuppressive cytokine IL-10 (186-188). The interaction between MUC16 and Siglec-9 likely leads to a reduced ability of NK cells to react against ovarian tumor targets. Based on the shielding effect of MUC16 and the interactions of the mucin with $Siglec\text{-}9^{pos}$ NK cells, we are proposing a model by which ovarian tumor cells are protected from immune attack (Figure 4) (109, 182). According to this model, MUC16 shed from the surface of the ovarian tumor cells binds to NK cells via Siglec-9. This receptor-ligand interaction occurs locally in the tumor microenvironment as well as systemically, as evidenced by the binding of the mucin in the peritoneal environment as well as in systemic circulation (42). The interaction between Siglec-9 and MUC16 provides an opportunity for the mucin to mediate suppression of NK cell cytolytic activity even before the NK cells engage the cancer cells. Siglec-9 engagement causes phosphorylation of tyrosine residue in the distal ITIM domain of this receptor followed by recruitment and activation of the phosphatases, SHP-1 and SHP-2 (187). As a result, the phosphorylation events (phosphorylation of Vav-1, WASp, and other proteins) necessary for the formation of an NK cell activating synapse are inhibited and hence the NK cells are unable to lyse tumor targets (96, 98, 189). Therefore, the selective expression of Siglec-9 on the $CD16^{pos}/CD56^{dim}$ NK cells likely leads to protection of the ovarian cancer cells from the cytolytic responses of a major innate immune cell subset (Figure 4).

Our data indicates that the ovarian tumor cells also utilize the molecular properties of MUC16 to protect themselves from the cytolytic responses of $Siglec\text{-}9^{neg}$ $CD16^{pos}/CD56^{dim}$ cytolytic NK cells. This is achieved via the shielding effect of MUC16 that hinders the ability of the $Siglec\text{-}9^{neg}$ NK cells to form activating immune synapses with the ovarian cancer cells.

There are two additional prominent examples of the immunoprotective effect afforded by sialylated glycans. The first example we will consider is glycodelin since this glycoprotein is also expressed by ovarian tumors (190). Glycodelin expression has been observed in immunohistological specimens of ovarian tumors as well as in the peritoneal fluid of cancer patients. In addition to being expressed by ovarian tumors, the major research on

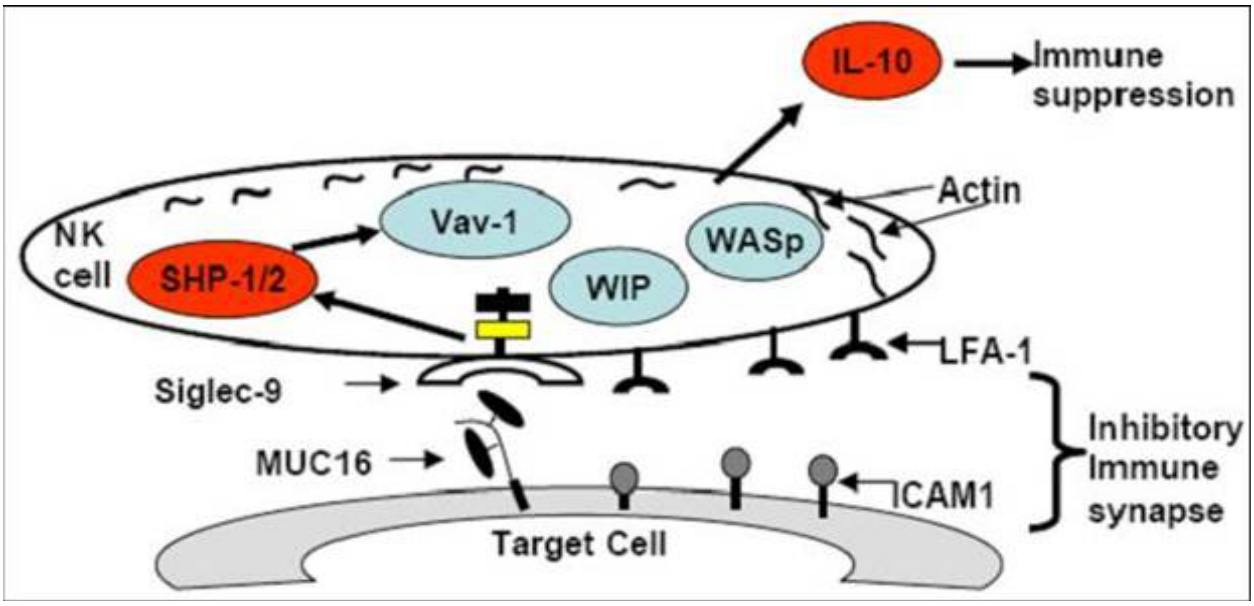

Figure 4. Model for MUC16 induced NK cell suppression. MUC16 binding to Siglec-9 leads to phosphorylation of proximal ITIM (yellow box) of the receptor and subsequently phosphorylation and activation of SHP-1 and/or SHP-2. SHP-1 and SHP-2 are phosphatases that dephosphorylate Vav-1 and other signaling molecules. As a result, polymerization of actin, polarization of actin associated proteins (WASp, WIP, Myosin II), LFA-1, and perforin granules is not achieved. Lack of activating synapse formation leads to protection of the tumor cell. csMUC16-Siglec-9 binding triggers an inhibitory immune synapse that protects the tumor cells from NK cell attack. MUC16 binding to Siglec-9 upregulates IL-10 and other Th2-type cytokines that inhibit the anti-tumor immune responses.

this molecule has been focused on its role in protecting the human embryo/fetus from the maternal immune responses. In this context, several reports have demonstrated the ability of glycodelin in regulating the immunologic function of dendritic cells, NK cells, and $CD4^{pos}$ and $CD8^{pos}$ T cells (162, 191-196). As mentioned previously, glycomic analysis of glycodelin from amniotic fluid revealed the presence of LacdiNAc glycans (159). However, the isoform of glycodelin expressed in human seminal fluid showed predominant expression of high mannose type and fucosylated N-glycans and a total absence of the LacdiNAc type oligosaccharides (197). Subsequent analysis of glycodelin isolated from other tissues and fluids revealed differences in the glycan expression profile of the different isoforms and no reported changes in the primary amino acid sequence of this glycoprotein (198). Therefore, the glycodelin isoforms have provided an excellent opportunity to reveal the biologic activities of oligosaccharides from the different tissue-specific isoforms of the molecule. It is now confirmed that glycodelin expressed in the amniotic fluid (glycodelin-A, Gd-A) and follicular fluid (Gd-F) modulate immune responses whereas glycodelins present in the seminal fluid (Gd-S) and the cumulus matrix (Gd-C) that surrounds the human oocyte are not immunomodulatory (198). These differences in the activities of the glycodelin isoforms likely arise from their different glycosylation patterns. Glycodelin isolated from the peritoneal fluid of ovarian cancer patients decreases dendritic cell maturation and induces increased expression of the immunosuppressive cytokine IL-10 (191). These results suggest that the ovarian cancer associated glycodelin likely possesses the appropriate glycosylation pattern that facilitates the immunomodulatory properties of this glycodelin.

Glycomic analysis of the Gd-A, Gd-F, Gd-S, and Gd-C isoforms indicated major differences in the glycosylation patterns of the glycodelins (198). Immunologic assays showed that only the Gd-A and Gd-F isoforms and not Gd-C or Gd-S inhibited Jurkat T cell proliferation, induced apoptosis in the cells, and impaired their ability to respond to IL-2 stimulation. Enzymatic desialylation of Gd-A reversed its suppressive effects on the Jurkat T cells indicating that the sialylation of glycoprotein may serve as an important factor in modulating immune responses against ovarian tumors (198).

Another line of evidence indicating the importance of sialylation in tumor immunology is a recent study in the methylcholanthrene induced fibrosarcoma model (199, 200). It was observed that methylcholanthrene induced fibrosarcomas grown in immunecompetent mice expressed high levels of sialic acid on their cell surface. However, the same type of tumors grown in IL-1$\square^{-/-}$ and IFN-$\square^{-/-}$ immunocompromised mice expressed lower levels of sialylation (199). Extensive analysis in this model resulted in the understanding that the immune cells were selectively lysing fibrosarcoma cells that were expressing low levels of sialic acids while those expressing high levels of sialic acid were being selectively excluded from immune cell attack. Selective elimination of the tumors expressing low levels of sialic acid is mediated by NK cells and is

associated with increase immune synapse formation and increased unmasking of the ligands for the NK cell activating receptor NKG2D. Thus degree of sialylation of the tumor cells was serving as an important factor that contributed to immunoediting in this model (199).

In this context it is also important to mention that in our experiments we have found that ovarian tumor cells surviving an initial NK cell challenge in in vitro experiments express higher levels of MUC16 (109). These examples suggest that a consequence of the immunomodulatory properties of glycoconjugates may be their direct contribution to the development of highly immune resistant tumors via the process of immunoediting.

7.3. Cytokine binding to glycoprotein

Cytokines are important mediators of the immune response. Their activities can be either stimulatory or inhibitory and are mediated via specific receptors expressed on defined subsets of immune cells. Typically, cytokines attach to their receptor via specific peptide epitopes. However, it has been proposed that in addition to their peptide binding epitopes, cytokines also possess novel carbohydrate recognition domains that allow them to recognize a variety of oligosaccharide ligands (201, 202). IL-1alpha IL-1beta, IL-2, IL-3, IL-4, IL-6, IL-7, and other cytokines have been shown to possess carbohydrate recognition domains that allow them to bind to specific oligosaccharides (201, 203-210). Cytokines bind to specific glycan sequences expressed on glycoproteins such as ribonuclease B, Tamm Horsfall glycoprotein and uromodulin (211-215). To the best of our knowledge, specific instances of ovarian cancer associated glycoproteins interacting with cytokines via their glycan chains have not been reported. However, mucins such as MUC1 and MUC16 have been postulated to modulate immune responses by scavenging cytokines and thereby hindering their ability to interact with the cytokine receptors on the immune cells (216). It is worth noting that IL-2 was shown to bind to high mannose type oligosaccharide ligands with binding constants in the micromolar range (212). However, a recent exhaustive study of IL-2 that used ELISA, chromatography, equilibrium dialysis, and NMR spectroscopic methods did not show any significant binding interaction between IL-2 and the high mannose chains (217). Thus the subject of cytokine-glycan interactions should be approached with caution.

7.4. Aberrant glycosylation and the autoantibody response

Cancer associated mucins are aberrantly glycosylated, as evidenced by the abundant expression of Tn and sialyl Tn antigens. In addition, mucins expressed by cancer cells are also not fully glycosylated leading to existence of peptide epitopes that are devoid of glycans (100, 103, 216, 218). The expression of such aberrant glycans and non-glycosylated peptide epitopes results in their detection by the immune system and the subsequent generation of an autoantibody response against these antigens. In the case of ovarian cancer, autoantibodies have been detected against MUC1 and MUC16 (219-226). The generation of humoral responses against the cancer associated antigens may imply another mechanism of active immune surveillance. A careful characterization of the aberrantly glycosylated epitopes of glycoproteins that generate a more robust autoantibody response will result in development of novel immunologic strategies to combat ovarian and other tumors. The use of dendritic cells loaded with MUC1 glycopeptides is another approach that may likely lead to better anti-tumor vaccine strategies (139, 140, 226, 227).

In the case of cancer patients, aberrant glycosylation is detected not only in the glycoproteins that are expressed by the tumors but also by proteins expressed by healthy tissues. A classic example is the development of antibodies in cancer patients with glycosylation profiles that are diverse from those found in antibodies developing in non-cancer patients. IgG fractions of ovarian cancer patients carry a higher percentage of the agalactosylated and core fucosylated N-linked oligosaccharide chains (228). Another group has demonstrated an approximately four-fold increase in the binding of the total IgG fraction to immobilized ConA as compared to IgG derived from the serum of healthy controls (38% versus 9%) (229). Greater than 87% of the IgGs that were reactive against ovarian cancer antigens bound to immobilized Con A. Such changes in glycosylation are observed not only on the antibodies but also on other serum glycoproteins including haptoglobin, alpha1-acid glycoprotein, and alpha1-antichymotrypsin (228, 230). It has been proposed that identification of the glycosylation alterations in ovarian cancer may be used to develop novel diagnostic tests for the detection and monitoring of this disease (230).

8. CONCLUSIONS

Glycoconjugates display redundant mechanisms for immunomodulation. The plasticity of the glycosylation process leads to development of glycoproteins and glycolipids that express a diverse array of glycans in healthy individuals versus cancer patients. The tumors likely utilize this plasticity to develop mechanisms to modulate immune responses that allow them to survive during the different stages of the development of cancer. Thus, the glycoproteins may allow them to withstand immune surveillance, contribute towards development of immune resistant tumors (as predicted by the immunoediting hypothesis), and in the advanced stages of tumorigenesis, may alter immune cell function and phenotype thus hijacking an important tumor control mechanism. Several different mechanisms of immunomodulation are displayed by ovarian tumors, indicating a very diverse and redundant strategy. The silver lining is that while such strategies may allow the cancer cells to overcome native immune responses, these mechanisms may not always be able to overpower immune responses induced via the application of immunologic anti-cancer agents. Thus, the administration of antibodies, cytokines and other strategies lead to development of strong immune responses against tumor cells and in some cases have also been associated with efficient cancer control. Indeed, in our own in vitro experiments we have

demonstrated that the immune shielding provided by MUC16 to ovarian cancer cells can be surpassed with administration of immunotherapeutic agents that lead to antibody mediated tumor cell death (231).

9. PERSPECTIVE

Several of the examples of glycoproteins discussed in this review are expressed not only by ovarian tumors but are also temporally expressed in the human endometrium and/or the human decidua where protection of the embryo/fetus from maternal immune responses is paramount. The role of glycoconjugates in suppressing the maternal immune responses has previously been elaborated (175, 232-234). We propose that ovarian, and quite possibly other tumors, are co-opting these already existing glycoprotein based strategies that are central to human reproduction, to effectively neutralize the immune system. It is important that we recognize the glycobiological connections between pregnancy and cancer to fully understand the immune system, a concept that was introduced several decades ago with the identification of onco-fetal antigens.

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