



Mucins as anti-cancer targets: perspectives of the glycobiologist

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Received: 14 January 2021 / Revised: 22 February 2021 / Accepted: 26 February 2021 / Published online: 11 March 2021
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Abstract

Mucins are highly O-glycosylated glycoproteins that carry a heterogenous variety of O-glycan structures. Tumor cells tend to overexpress specific mucins, such as the cell surface mucins MUC1 and MUC4 that are engaged in signaling and cell growth, and exhibit abnormal glycosylation. In particular, the Tn and T antigens and their sialylated forms are common in cancer mucins. We review herein methods chosen to use cancer-associated glycans and mucins as targets for the design of anti-cancer immunotherapies. Mucin peptides from the glycosylated and transmembrane domains have been combined with immune-stimulating adjuvants in a wide variety of approaches to produce anti-tumor antibodies and vaccines. These mucin conjugates have been tested on cancer cells in vitro and in mice with significant successes in stimulating anti-tumor responses. The clinical trials in humans, however, have shown limited success in extending survival. It seems critical that the individual-specific epitope expression of cancer mucins is considered in future therapies to result in lasting anti-tumor responses.

Keywords Mucin · MUC1 · MUC4 · O-glycans · Glycopeptides · Epitopes · Immunotherapy · Anti-mucin antibodies · Cancer · Vaccine

Introduction

Cancer is a leading cause of death worldwide, and the majority of cancer deaths is due to the formation of metastases. Cancer treatments include anti-hormone receptor analogs, cytostatic drugs, immunotherapy and vaccines. Although many different treatment options exist, the side effects are often daunting and the hope is for safe, individual-specific and effective methods of immune stimulation to mobilize the rejection of tumor cells. Multiple cancer-associated tumor antigens have been identified and proposed as diagnostic markers and therapeutic targets. This includes mucins, mucin-like glycoproteins and glycoproteins that are overexpressed in tumors. Tumor-associated glycans include the Tn and T (Thomsen-

Friedenreich) antigens, Lewis antigens and a number of gangliosides.

Cancer-associated glycans in cancer vaccines are in context with proteins that are overexpressed in cancer. Thus, vaccines should not target O-glycans or N-glycans that are found on normal glycoproteins. Stem cells are critical for tumor development and mucin glycopeptides found specifically on stem cells would be ideal targets to stop tumor growth. Although additional cancer therapy may aid in the effectiveness of vaccines, mucin-based vaccines are likely to be more successful in patients that do not undergo immunosuppressive treatments.

Glycoproteins and large mucins can carry O-glycans O-linked to Ser/Thr or N-glycans that are N-linked to Asn residues. In tumor cells, the O-glycans are often truncated and highly sialylated, while N-glycans can be altered in their branching patterns and exposure epitopes such as the Lewis antigens. The mechanisms of these alterations include the up- and downregulation of the expression of glycosyltransferases (GTs) involved in the synthesis of glycans [1]. However, very little is known about the control of GT gene expression in cancer cells. Some of the specific factors modifying GT activities are cytokines and growth factors.

Glycoproteins that are overexpressed in tumor cells and can serve as markers or targets include human epidermal growth factor receptor2 (HER2), mucins MUC1, MUC4 (Fig. 1) and

Glycoconjugate Journal: special issue on Glycoconjugate vaccines: classic and novel approaches.

Editor: Roberto Adamo, December 31, 2020, <https://www.springer.com/journal/10719>

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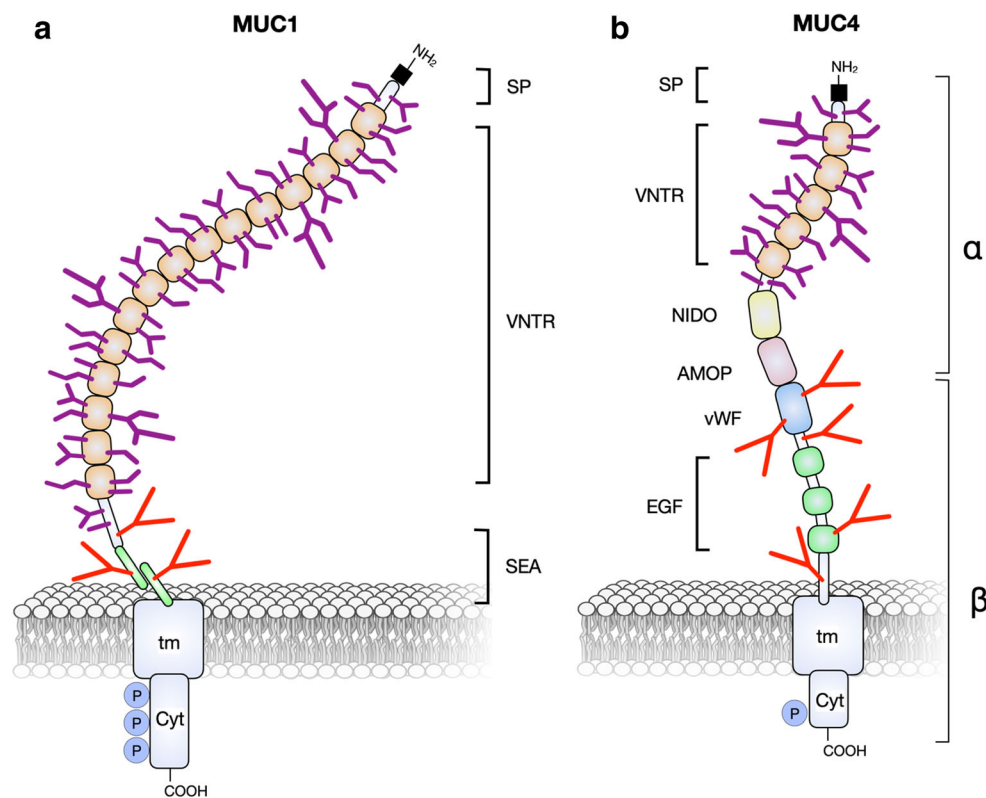


Fig. 1 Cell surface mucins MUC1 and MUC4. **a** MUC1 contains a C-terminal cytoplasmic domain (Cyt) with several phosphorylation sites (P). The transmembrane domain (tm) spans the membrane and is continuous with extracellular sea urchin sperm protein, enterokinase and agrin (SEA) domain, the site for proteolytic cleavage of the mucin. Potential N-glycosylation sites are indicated by red “Y” glycan structures. The majority of MUC1 is composed of variable number tandem repeats (VNTR) which are heavily O-glycosylated. O-glycans are shown in purple. MUC1

terminates in an N-terminal signal peptide (SP). **b** MUC4 is composed of two subunits: N-terminal α domain and C-terminal β domain. MUC4 has a similar C terminus to MUC1 but having only one phosphorylation site. The tm is linked to epidermal growth factor (EGF) domains and von Willebrand factor (vWF) domain containing several potential N-glycosylation sites. The α subunit is composed of an adhesion-associated domain (AMOP), nidogen (NIDO)-like domain, O-glycosylated VNTR and N-terminal SP.

MUC16, epithelial-cell adhesion molecule (EpcAM) and prostate-specific antigen (PSA). A proportion of cell surface-bound glycoproteins is cleaved and can interact with antibodies in the serum. Carbohydrate-binding proteins (lectins) on immune cells and tumors facilitate interactions within the tumor microenvironment (TME) and cancer cell adhesion, thereby promoting cancer progression and metastasis. Sialic acid-binding lectins (Siglecs) expressed on specific hematopoietic cells such as natural killer (NK) cells and macrophages regulate immune responses while soluble Gal-binding galectins are involved in cell proliferation and apoptosis. Selectins expressed on endothelial and immune cells adhere to Lewis-type glycans expressed on tumor cells and promote their invasion and dissemination. Cancer cells that detach from the tumor enter the blood or lymph and can form metastases. The composition of the cancer environment, the extracellular matrix (ECM) and TME control the diffusion of immune cells to the tumor and act in an immunosuppressive fashion. The antigenicity of tumor epitopes is in constant flux and it is important to search for key targets involved in tumor growth without eliciting cross-reactivity. Vaccine preparations containing conjugates of non-

human mucins or cancer-associated carbohydrates have been tested since 1992, but with minimal success in cancer treatment and prevention. In order to confirm the expression of mucins on tumors, anti-mucin antibodies could be used to label biopsies or tumors in vivo. Efforts have been made to produce safe anti-cancer vaccines with high specificity and efficacy that can reduce tumor growth [2].

Microbiota function in detoxification and maintaining a balance in host cell growth and proliferation. While microbes including viruses can trigger amplification of genes involved in cancer progression and immunosuppression, they are also involved in the stimulation of the immune system and host responses [3]. Epithelial cell mucins bind to microbes but also form a barrier against pathogens. Studies in mice show that the microbiota collaborate with the immune system in anti-tumor responses. For example, killed *Streptococci* and *Serratia* have been used in immunotherapy. Live bacteria in the bladder (Bacillus Calmette-Guérin, BCG) trigger immune responses against the tumor cells and are being used to successfully treat invasive bladder cancer [4]. Immune cells with roles in supporting BCG therapy include $CD4^+$ and $CD8^+$ lymphocytes,

NK cells, macrophages and dendritic cells (DC). Bladder cancer cells are killed through direct cytotoxicity by these immune cells and by soluble apoptosis-inducing factors such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). BCG is able to stimulate anti-tumor immune responses in patients with bladder cancers that highly express sialyl-Tn and sialyl α 2–6(Gal β 1–3)GalNAc- antigens (Fig. 2) [5].

This review describes the approaches taken for the design of vaccines based on epitopes found on cell surface mucins of cancer cells with a focus on the major mucin targets MUC1 and MUC4. The goal is to stimulate safe, natural defenses against tumor cells.

Mucins

Mucins are expressed on epithelial and goblet cells and are targets for immunotherapy because of their overexpression and abnormal glycosylation patterns in a variety of different tumors [6]. The secreted mucins include small mucins (MUC7 and 8), large polymeric mucins (MUC2, 5 AC, 5B, 6, 19) and the cell surface-bound signaling mucins (MUC1, 3, 4, 12, 13, 15, 16, 17, 20, 21, 22) as well as their circulating cleavage products that are of particular importance for cancer. Mucins have variable number of tandem repeat regions (VNTR) that are densely O-glycosylated with GalNAc O-glycans attached while N-glycosylation sites are found mostly outside of this region. Mucins are extremely large glycoproteins but are

functionally diverse with distinct domains. While soluble mucins are thought to function in the protection of the epithelium, the roles of cell surface mucins include cell adhesion, receptors for glycan binding proteins, interaction with other cell surface receptors, regulation of the immune system as well as cell signaling, growth and proliferation, and are thus of particular importance for the biology of tumor cells. Mucins bind to mammalian lectins such as siglecs expressed on DC and interact with platelets, neutrophils and macrophages via specific glycans [7]. These interactions regulate the immune responses against tumors. Infections and chronic inflammation also play a role in tumorigenesis [8]. For example, Toll-like receptor 4 (TLR4) is activated by bacteria leading to the production of inflammatory cytokines. Mucins also bind to TLR4 thus controlling inflammation, while inflammatory cytokines can regulate both mucin expression and glycosylation [9, 10].

The cell surface mucin MUC1 has been found to be dramatically increased in most tumor cells studied [11]. The VNTR domain of MUC1 has multiple Pro/Ser/Thr-rich sequences (PDTRPAPGSTAPPAGVTS), each carrying up to five O-glycans. The potential of MUC1 as a vaccine candidate is demonstrated by the fact that natural immunity against MUC1 has been found in cancer patients. These naturally occurring polyclonal antibodies bind to a variety of mucin epitopes [12]. The anti-Tn antibodies isolated by GalNAc-affinity chromatography were shown to bind to a great variety

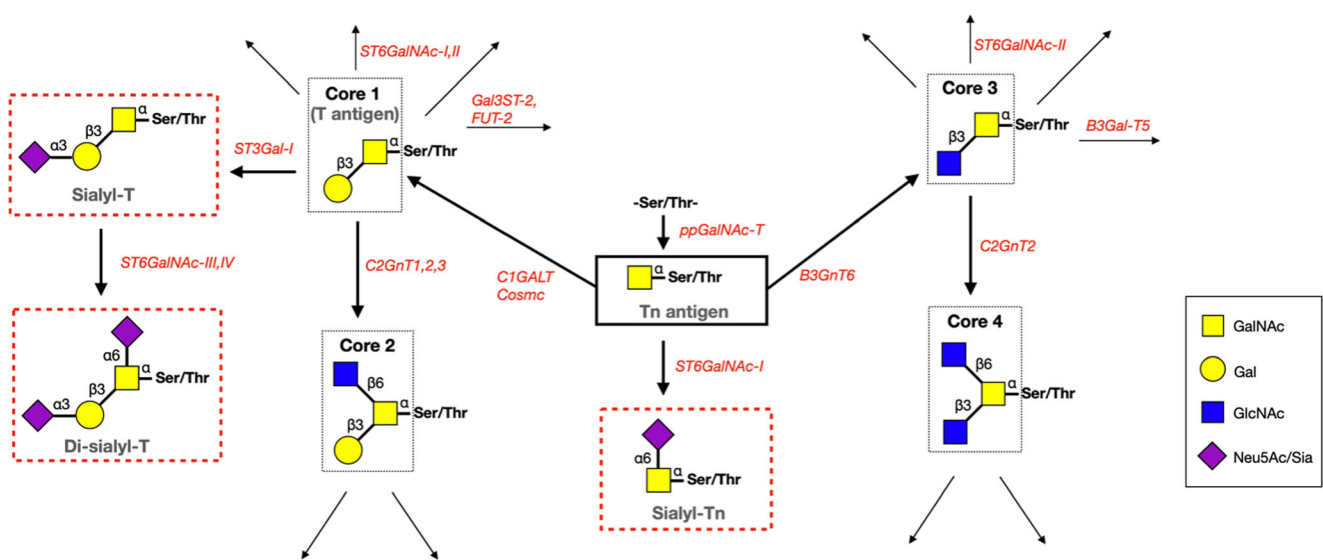


Fig. 2 Synthesis of cancer-associated O-glycans. Ser/Thr residues of mucin peptides are O-glycosylated by one of several ppGalNAc-Ts that transfer GalNAc in an α linkage to form the Tn antigen. The Tn antigen can be converted to many different O-glycan structures, and GTs compete for the available acceptor substrates. Tn antigen can be sialylated by ST6GalNAc-I but can also be converted to core 1 (T antigen) by β 3 Gal-transferase C1GALT (with coexpressed chaperone Cosmc) or to core 3 by β 3 GlcNAc-transferase B3GnT6. T antigen is converted to core 2 via the addition of a β 6-GlcNAc residue by C2GnT1,2,3. However, it can

also be converted by several other GTs including ST3Gal-I and ST6GalNAc-III,IV, Fuc-transferase FUT2 and sulfotransferase Gal3ST-2. Core 3 is converted to core 4 by β 6 GlcNAc-T C2GnT2, or is galactosylated (by B3Gal-T5) or sialylated. Core 2, 3, 4 structures can be extended by Gal- and GlcNAc-transferases in various pathways in the synthesis of antigens such as fucosylated blood group and Lewis antigens. The structures in cancer are mostly truncated and sialylated Tn and T antigens

of GalNAc-containing oligosaccharides and GalNAc-peptide epitopes.

Both anti-MUC1 IgG and IgM antibodies were found in breast and gastric cancer, and the levels of antibodies correlated with increased survival of patients [13–15].

The extracellular O-glycosylated domain of cell surface mucins can be released and found in the circulation which may interfere with mucin immunotherapy. Autocleavage sites are near the membrane and in several mucins include the SEA (sea urchin sperm protein, enterokinase, and agrin) domain. After cleavage, the N-terminal (β) membrane-bound and the C-terminal (α) O-glycosylated domains (Fig. 1) form a non-covalently linked heterodimer [16]. The cytoplasmic tail of MUC1 (MUC1-C) has been well characterized and facilitates their dimerization through disulfide bonds involving the CQC sequence. There are five N-glycosylation sites of MUC1 close to the transmembrane domain [17].

The C-terminal cytoplasmic domain of MUC1 is involved in signal transduction and regulating cell growth, differentiation and survival signals. It can be phosphorylated, associate with galectin-3, block apoptosis and has been named ‘oncogenic’ MUC1-C or ‘oncoprotein’. Multiple Tyr, Thr and Ser residues in the cytoplasmic domain are phosphorylation sites and can activate signaling pathways involving Ras and catenins. The epidermal growth factor (EGF) domains of cell surface-bound mucins form complexes with other proteins, e.g. HER2 Tyrosine kinase. MUC1 also associates with the oncogene product p53. MUC1-C forms complexes with transcription factors, and translocates to the nucleus by an unknown mechanism, where it is believed to influence gene transcription. MUC1-C has also been proposed to attenuate apoptotic pathways and increase resistance to apoptosis-inducing drugs [6, 17, 18]. A number of different cancer cells have been examined for the effect of blocking dimerization of MUC1-C. The cell-penetrating peptide GO-203 blocks MUC1-C homodimerization leading to a reduction in the oncogenic potential of MUC1-C in cultured cancer cells and in mice [19–22].

MUC4 is another cell surface mucin that is increased in pancreatic, breast, lung and ovarian tumors. Especially in pancreatic cancer, MUC4 is expressed at high levels while it is almost undetectable in normal pancreatic tissue [23]. MUC4 has highly O-glycosylated VNTR and also multiple N-glycosylation sites near the EGF domains and within the von Willebrand factor (vWF) domain (Fig. 1). The nidogen (NIDO)-like domain, adhesion associated domain (AMOP) present in some splice variants and vWD type D domains synergistically participate in MUC4 function in cancer [24, 25]. MUC4 interacts with other proteins and is involved in cell signaling. Thus, MUC4 is a tumor marker as well as a target for immunotherapy [26]. MUC4 is autocatalytically cleaved releasing the O-glycosylated α domain from the β domain (Fig. 1). MUC4 is thought to promote tumorigenicity

and aggressive behavior of tumors, and its high expression correlates with poor outcomes in various types of epithelial carcinomas. Both MUC1 and MUC4 play a role in the progression and metastasis formation of pancreatic cancer.

The cell surface mucin MUC16 is known as the ovarian cancer antigen CA 125. MUC16 is also overexpressed in pancreatic cancer and accompanied by MUC16 antigen-specific T cell clones, and could serve as a potential target for developing cancer immunotherapy [7, 23, 27]. In addition, secreted MUC5AC is found in the serum of pancreatic cancer patients and can be used for diagnosis. Thus, MUC1, MUC4, MUC5AC and MUC16 play a role in disease progression in pancreatic cancer [28] and are valuable diagnostic biomarkers, especially in combination with the expression of sialyl-Lewis^a glycans [29]. The expression of large polymeric, secreted mucins has been studied in a number of tissues, cells and cancer types and these mucins have been related to tumor biology [30]. There is no clear correlation between the expression of specific mucins with malignant potential and tumor progression [31]. It appears though that the ratio of mucins expressed varies between tissues and cancer types and in different patients, but may nevertheless be useful in determining the primary site of metastatic tumors. The expression of soluble MUC2, MUC6 and MUC5A mucins has been found to be down regulated in gastric cancer, while MUC2 and MUC5AC expression was up regulated in colorectal cancer [32]. It was revealed that the expression of MUC2 in colorectal cancer was associated with a poor prognosis while MUC5AC and MUC6 expression could predict a more favorable prognosis [33].

Mechanisms of aberrant mucin glycosylation in cancer cells

Mucins can carry hundreds of O-glycan chains with different structures. The glycan structures are variable between glycosylation sites - and between mucins within a tumor - and are a result of complex regulation of GT activities [1]. A common tumor-associated glycan is the unmodified GalNAc α -Ser/Thr (Tn antigen), sialyl α 2-6GalNAc (sialyl-Tn antigen) and the unmodified T antigen, O-glycan core 1 (Gal β 1-3GalNAc) and its sialylated versions (Fig. 2). The sialyl-T antigens are not cancer-specific but can regulate overall O-glycosylation [34], cellular interactions and cell death [35]. In addition, more complex neutral, sialylated and fucosylated glycans and those carrying Lewis epitopes are often found in increased amounts in O-glycans, N-glycans or glycolipids and have been proposed as biomarkers [36]. The mechanisms of these changes include the up- or downregulation of GTs, possible relocalization of GTs within the Golgi stacks and availability of donor and mucin acceptor substrates. Enzymes often compete for the same acceptor substrate and the relative GT activities determine the final outcome.

The first sugar of all O-glycans, GalNAc (Tn antigen), is normally substituted by Gal β 1-3 (in core 1) or by GlcNAc β 1-

3 (in core 3). Twenty different polypeptide GalNAc-transferases (ppGalNAc-Ts) synthesize the Tn antigen and contribute to differentiation, cancer development and metastasis. These enzymes differ in function, depending on the cell type and isoform [37, 38]. Several of these enzymes have been shown to be aberrantly expressed in cancer and involved in cancer progression. In hepatocellular carcinoma, mainly ppGalNAc-T1 and 2 are expressed. Overexpression of ppGalNAc-T2 blocks Tyr phosphorylation of the EGF receptor and reduces tumor growth in mice [39]. Pancreatic tumors highly express ppGalNAc-T3, while downregulation of the gene induces apoptosis and decreases tumorigenicity of pancreatic cancer cells [40]. ppGalNAc-T4 is highly expressed in breast cancer and is involved in the regulation of estrogen levels in breast cancer cell lines [41]. Another enzyme, ppGalNAc-T6, is particularly well expressed in breast tumors and is used as a biomarker [42]. The gene encoding ppGalNAc-T6 is frequently methylated at later stages of cancer but infrequently methylated in primary breast tumors. The ppGalNAc-T9 is a brain-specific enzyme that appears to be involved in brain metastases of breast tumors [43]. Overexpression of ppGalNAc-T14 in breast cancer cells MCF-7 indicates the critical role of ppGalNAc-T14 in cell invasion and migration [44]. Knockdown of ppGalNAc-T14 in MCF-7 cells affected the gene expression patterns of a number of proteins and decreased the expression of MUC1. Overexpression of ppGalNAc-Ts, or possibly their abnormal localization within the Golgi membrane assembly line, might increase the amount of unmodified GalNAc residues.

Cell surface glycosylation has been shown to control tumorigenesis and metastasis [45]. The Tn antigen has been correlated with metastasis and poor outcomes in cancer patients [46]. The lack of further processing of GalNAc to more complex chains could be due to a downregulation of core 1 β 1,3-Gal-transferase (C1GalT) or core 3 β 3-GlcNAc-transferase (B3GnT6) (Fig. 2). C1GalT activity may also be absent due to the lack of co-expression of its specific chaperone Cosmc [47]. The T antigen is normally converted to branched and elongated O-glycans. However, low activities of specific GlcNAc-, sialyl- or Fuc-transferases that modify core 1 result in the overexpression of the T antigen in cancer cells.

The sialylation of GalNAc to form the cancer-associated Sialyl-Tn antigen will also prevent the synthesis of core 1. Overexpression of α 2,6-sialyl-transferases ST6GalNAc-I [48] and ST6GalNAc-II [49] has been observed in tumor cells that display the sialyl-Tn epitope. ST6GalNAc-I is the main enzyme that synthesizes and correlates with sialyl-Tn [50]. However, the sialic acid residue can be O-acetylated which would prevent binding of antibodies to the cryptic sialyl-Tn [51]. ST6GalNAc-I was found to contribute to cell proliferation and invasion of mouse hepatocarcinoma cancer cells [52] and to tumor growth of breast cancer cells in mice [48].

In normal lung and intestinal tissues, the Tn antigen is converted to core 3 by B3GnT6 which competes with C1GalT for

GalNAc-acceptor substrates [53]. A downregulation of the B3GnT6 gene expression and activity has been observed in cancer cells and tumors [54–56] and allows the increased synthesis of core 1 (T antigen) by C1GalT. Colon cancer cells that overexpress B3GnT6 exhibit a significantly reduced metastatic potential in nude mice. This suggests that the expression of B3GnT6 or the presence of core 3 in glycoproteins is a marker of healthy tissue. Deletion of the B3GnT6 gene in mice led to increased permeability of the intestinal barrier and increased susceptibility to colitis and colorectal adenocarcinoma [57]. B3GnT6 is not expressed in normal human pancreatic epithelial cells but is expressed in many of the pancreatic ductal adenocarcinoma and cancer cells, correlating with the expression of MUC5A [58]. The expression of B3GnT6 increased with differentiation towards gastric mucus-producing cells and may be a favorable prognostic factor. Re-expression of B3GnT6 in pancreatic cancer cells suppressed tumor growth, migration and invasion through matrigel in vitro. Mice injected with B3GnT6-expressing pancreatic cancer cells showed significantly lower tumor growth and metastasis. The mechanisms by which core 3 exerts its anti-tumor function remain to be established.

The branched core 2 structure, GlcNAc β 1–6(Gal β 1–3)GalNAc-, can be synthesized from core 1 by three variants of C2GnT. C2GnT1 is variably active in cancer cells. In certain metastatic prostate cancer cells, C2GnT1 activity is present at high, low or undetectable levels [59]. The α 2,3-sialyltransferase ST3Gal I also acts on core 1 and competes with C2GnT1. Many cancer cells have a high activity of ST3Gal I, and the enzyme controls cancer cell proliferation. The overexpression of ST3Gal I in mice is related to increased breast tumor growth [60] and downregulation of the enzyme reduced prostate tumor growth [35]. This may be due to the protective function of sialic acid that prevents removal of cells through anti-T antigen immunity. The synthesis of Sialyl α 2–3Gal β 1–3GalNAc- by ST3Gal I in breast cancer cells and tumors [61] prevents the conversion of core 1 to core 2 and to complex O-glycans, although ST6GalNAc-III and IV can add a second sialic acid residue to core 1. As a consequence, breast cancer cells exhibit mainly short, sialylated O-glycans [34]. These smaller O-glycans attached to MUC1 allow the binding of anti-MUC1 peptide antibodies directed against normally cryptic peptide epitopes. Thus, the relative activities of competing GTs shape the glycan structures and control epitope exposure of mucins in cancer cells. This phenomenon is important for the design of a mucin-based cancer vaccine.

O-glycan core 3 can be converted to core 4 by C2GnT2 [62, 63]. Although both core 3 and 4 are restricted in their tissue expression, C2GnT2 is expressed in a number of colon and lung cancer cells with relatively low activity. Cytokines interleukin-4 and interleukin-13, and retinoic acid upregulate C2GnT2 activity in lung cancer cells [64]. The overexpression of C2GnT2 in HCT116 colon cancer cells resulted in decreased growth, cell

adhesion and invasive properties [65]. In addition, overexpression of C2GnT2 in nude mice reduced tumor growth. This indicates that both core 3 and core 4 structures of mucins are associated with low tumorigenicity of cancer cells.

Gastric gland mucins express O-glycans with terminal GlcNAc α 1–4Gal- epitopes that are primarily attached to MUC6. This epitope is also common in premalignant conditions such as pyloric gland adenoma of the stomach, chronic atrophic gastritis, Barrett's esophagus and pancreatic intraductal papillary-mucinous neoplasms. The gene encoding α 1,4-GlcNAc-transferase that synthesizes the GlcNAc α 1–4 epitope is highly expressed and the enzyme is another marker for pancreatic cancer and other cancers [66].

Selection and immunogenicity of mucin epitopes

Knowledge of the molecular basis that governs epitope presentation by immune cells, as well as interactions between antigens and elicited antibodies is limited. Nuclear magnetic resonance spectroscopy of mucin peptide and glycopeptide antigens in solution as well as crystallization studies are excellent methods to determine antigen folding and conformations [67]. Molecular dynamics simulations are also helpful to determine the spatial orientation of glycan epitopes in mucins and to anticipate their binding abilities and antigenicity [68].

The glycosylation and conformations of MUC1 peptides strongly affect antibody binding [61, 67, 69, 70]. Thus, for the generation of high affinity therapeutic antibodies, both the peptide and the glycans need to be considered in context. Antibodies that bind to mucin peptides on cell surfaces can cause clustering of mucins, thus enhancing immune recognition or cell adhesion. This mucin clustering affects the exposure of tumor-associated carbohydrate antigens. Short glycans such as the Tn antigens generally exhibit low immunogenicity and have to be presented as clusters in order to produce effective mucin epitopes. However, Tn antigen attached to the Thr residue in the PDTRP sequence of MUC1 was used as a vaccine which led to an effective immune response without adjuvant and induced antibodies recognizing human breast cancer cells [71]. Antibodies recognize a spatial epitope which is difficult to predict due to the great variety of glycans, their complexity, large size and heterogeneous sugar composition and linkages but in silico modeling of epitopes has been attempted [72].

The synthesis of tumor epitopes has been highly successful for vaccine design [67, 73–75]. MUC1 peptides glycosylated with Tn and T antigens and their sialylated versions were synthesized and linked to tetanus toxoid peptide (T cell epitope). Dendrimers containing more than one MUC1 (glyco)peptide chain were shown to produce high antibody titers in mice. Multivalent constructs of mucin vaccines included glycosphingolipid GM2, glycopeptide from MUC1, and glycans Globo H, Lewis^Y, Tn and T antigens conjugated to KLH (*Keyhole limpet hemocyanin*), as

well as a purified tree bark extract adjuvant (QS-21) [76]. The vaccine was administered to prostate cancer patients and was found to be safe while raising IgM antibodies against Globo-H expressed on prostate tumors. The increase in PSA levels seen prior to vaccination was in part inhibited by the vaccine.

In order to create novel epitopes, MUC1 peptides carrying Sialyl-Tn antigen were linked to a mutagenized tetanus toxoid adjuvant to immunize mice as a preclinical breast cancer model. The vaccine showed immune responses, decreased tumor growth and increased survival [77]. KLH is another common adjuvant used in mucin vaccines [78]. While adjuvants are critical for an effective immune response, there are also self-adjuncting vaccines where an immune stimulant is present in the same molecule as the antigen. This includes MUC1 linked to lipopeptides that provide amphipathic properties to the antigen to improve the in vivo efficacy.

Chemical synthesis permits the introduction of antigenic epitopes into the antigen. A novel Tn antigen mimic composed of GalNAc attached to α -methylserine in glycopeptides appeared to retain the bioactive conformation recognized by anti-MUC1 antibodies [67, 79]. This vaccine elicited high antibody titers in mice [80, 81]. A modified T antigen (3-F-Gal β 1–3GalNAc-) linked to Thr in glycopeptides showed highly specific binding of anti-MUC antibody [82].

A monoclonal IgG1 antibody (PankoMab-GEX) recognizes a conformational epitope of MUC1 carrying Tn or T antigens. The antibody is safe, well tolerated, and showed promising anti-tumor recognition at higher dosages in patients with advanced disease. Adverse events in a small number of patients included diarrhea, intestinal perforation, peritonitis and tinnitus. However, further studies are required to evaluate the efficacy of PankoMab-GEX as a maintenance therapy in advanced cancers [83].

Immunotherapy

Cancer development is generally accompanied by naturally acquired IgG antibodies against tumor-associated epitopes, suggesting that natural immunity against tumors exists and could possibly be enhanced. Immunotherapy approaches are effective and safe strategies to increase natural immune defenses against cancer cells by therapeutic antibodies, immune checkpoint inhibitors, immune cell modulators and anti-cancer vaccines.

The scarcity and often poor antigenicity of cancer antigens and an immunosuppressive tumor microenvironment (TME) limits the success of immunotherapy in cancer [23]. The TME can favor the proliferation and metastasis of cancer cells, partly due to the presence of fibroblasts, regulatory T cells and macrophages and production of ECM (glyco) proteins like collagens and fibronectin, as well as cytokines secreted by immune or cancer cells. The ECM forms a physical barrier for T cell infiltration. Immunotherapy for cancer therefore

should address the immunosuppressive properties of the ECM, stimulate the immune responses, facilitate immune cell infiltration of ECM and tumors, and recognize specific tumor epitopes.

Monoclonal antibodies used in passive immunotherapy are designed to bind to specific tumor epitopes. Mucin antigens should be selected according to their tumor-specific expression and the ability to elicit strong immune responses. These antibodies are raised by immunization of mice either with tumor extracts or with purified or synthetic tumor mucin epitopes. Monoclonal antibodies raised against MUC1 carrying Tn and T antigens successfully elicited immune responses in mice with a number of different cancers. Humanized antibodies that contain a human antibody scaffold with the complementarity-determining region specific for tumor antigen binding have potential for treatment in humans. A number of these anti-tumor antibodies are being tested in preclinical and clinical trials [84]. Unfortunately, mucin fragments of MUC1 are present in the serum in high amounts at late stages of cancer. These soluble mucins can compete for the binding sites of anti-MUC1 antibodies, thus decreasing their binding to tumors. Other problems are the poor penetration of antibodies into the tumor and the heterogeneity of epitopes within the tumor (Table 1).

A novel approach of immunotherapy is using chimeric antigen receptor (CAR) T cells that are engineered to potently target tumor-associated glycosylation. A monoclonal antibody specific for MUC1 carrying Tn antigens was used to construct CAR T cells [95]. These T cells successfully decreased tumor growth in T cell leukemia and pancreatic cancer mouse models and specifically targeted tumor cells.

Immune checkpoints play significant roles in downregulating the immune responses and are targeted in cancer immunotherapy. More than 60 different immune checkpoints targets, including proteins and reference antibodies could be targeted. Many of these constitute cell surface glycoproteins including galectins and siglecs involved in the control of the immune system. Immune checkpoint inhibitors allow enhanced immune responses to cancer. The cytotoxic T lymphocyte-associated protein 4 (CD152, CTLA-4) is one of the checkpoint receptors on activated T cells. Anti-CTLA-4 antibodies have been successful in stimulating anti-cancer responses and tumor regression in patients [85]. Another checkpoint is the activity of the programmed death receptor PD1 that inhibits T cell responses and promotes apoptosis in T cells. Inhibitors of PD-1 and its ligand PD-1 L can reduce apoptotic signals in T cells and have been used in the treatment of melanoma, lymphoma and a number of other cancer types. Anti-PD1 antibodies given to cancer patients prolonged overall survival [86]. The function of T cell immunoglobulin mucins (TIM-3 and TIM-4) is similar to that of PD-1 resulting in apoptosis. TIM-3 has a mucin domain and is an immune checkpoint that regulates T cell responses [96].

Table 1 Development of mucin therapies in clinical trials

Immunotherapy	Cancer	Observation	Reference
Tn antigen, MUC1 vaccine	Breast cancer	Induces anti-breast cancer antibodies.	[71]
Multivalent vaccine: GM2, MUC1, Globo H, Lewis ^Y , Tn and T antigens / KLH, QS-25	Prostate cancer	Induces IGM against Globo H on prostate tumors.	[76]
PankoMab-GEX (monoclonal IgG1)	Multiple cancers	Recognizes T and Tn antigens of MUC1. Anti-tumor recognition, some clinical benefits.	[83]
Anti-CTLA-4 checkpoint antibodies	Many cancers, metastatic Melanoma	Anti-cancer responses, tumor regression, increased survival.	[85]
Anti-PD1 checkpoint antibodies	Melanoma, lymphoma, head and neck squamous cell carcinoma	Prolonged overall survival, anti-tumor responses.	[86]
MUC1 peptide-primed DC	Pancreatic cancer	Reduced tumor load and growth.	[87]
Tecemotide-MUC1	Stage III lung cancer	No increase in survival. Successful in patients receiving concurrent chemotherapy and radiotherapy.	[88]
Tecemotide-MUC1	HER2+ Breast cancer	Safe but no improvement to tumor burden.	[89]
TG4010 vaccine (vaccinia virus-MUC1, cytokines)	Metastatic renal clear-cell cancer	Extended survival in patients developing CD8 ⁺ T cell responses against MUC1, minimal side effects.	[90]
Vaccine of DC loaded with Tn-MUC1	Castrate-resistant prostate cancer	Phase I/II trial: Significant T-cell responses, safe, but limited trial.	[91]
Theratope (Sialyl-Tn linked to KLH)	Metastatic breast cancer	Phase II/III trial: Increased survival and prevention of relapse, anti-Sialyl-Tn mucin	[92]

Table 1 (continued)

Immunotherapy	Cancer	Observation	Reference
ImMucin, MUC1 signal peptide vaccine with GM-CSF	Multiple myeloma	responses, minimal toxicity. Phase I/II trial: safe vaccine, elicited antibodies and cell-mediated immunity, stabilizing disease.	[93]
Vaccine of MUC1 expressing DC, autologous cytotoxic T cells and gemcitabine	Late-stage pancreatic cancer	Safe immunotherapy, significantly more stable disease and higher survival rate.	[94]

TIM-3 is activated by galectin-9 and mediates CD8⁺ T cell exhaustion and controls macrophage activation that reduces the inflammatory responses [97]. Mice with B16-induced melanomas were vaccinated with irradiated B16 melanoma cells that express FLt3L, a cytokine ligand of Tyrosine kinase FLt3 that induces signaling and increased immune cell numbers. When mice were injected with monoclonal antibodies against checkpoints TIM-3 or TIM-4, the therapeutic effects of the B16 vaccine were enhanced and the numbers of NK cells infiltrating the tumors were increased.

Several tumors including neuroblastoma, small-cell lung cancer, melanoma, glioma and sarcomas display abundant glycosphingolipids on their cell surfaces, including ganglioside GD2. The combination of mucin antigens and ganglioside epitopes could afford enhanced responses although anti-GD2 treatment could potentially lead to side effects related to the function of GD2 in normal cells. Monoclonal antibodies and bispecific antibodies against GD2 have been used in cancer treatment with limited success [98]. Other combinations of bispecific antibodies against mucins and cell surface receptors such as CD3 or CD28 have also been successful in slowing down tumor growth in mice [99].

Tumor suppression in mice has also been achieved by injection of cDNA encoding human MUC1 [100]. Tumors were induced in mice 5 days after the cDNA injection with mouse tumor cells MC38, and most mice with prior cDNA treatment were free of tumors. The anti-tumor protection lasted for several months.

Anti-cancer vaccine preparations require the design of antigens, adjuvants and carriers to deliver the antigens and cytotoxic T cell epitopes [98]. These anti-cancer vaccines are based on a specific protein, glycan or glycopeptide antigen of cancer cells to stimulate the immune system to attack the tumor. The epitope that is recognized by antibodies and T cells

should be exposed and accessible on the cancer cell surface. Preliminary structural analyses of individual tumor antigens should identify the glycan chains and mucins present. The various types of vaccines developed include mucin glycopeptides, peptides of VNTR domains, signal peptides, C-terminal membrane-bound peptides (oncoprotein), or MUC1 DNA in a eukaryotic expression vector, and recombinant adenovirus or vaccinia virus encoding mucin peptides. The extracellular O-glycosylated domains of mucins are good candidates for cancer-specific vaccines.

DC play important roles in tumor development and are instrumental in engulfing vaccine epitopes. They interact between the innate and adaptive immune systems. DC have been primed with tumor associated epitopes or transfected with mucin cDNA in order to be more effective antigen presenters. DCs transfected with mRNA encoding MUC4 elicited strong CTL response in vitro in pancreatic ductal adenocarcinoma. Using MUC4 specific epitopes transduced into dendritic cells with universal DR-restricted T-helper epitope, a potent MUC4-specific cytotoxic T cell response was observed [101].

Several problems are inherent in immunotherapy and vaccines directed against glycosylated mucins: a) The glycan structures of mucins are heterogeneous and variable; b) both the peptides and the glycans of mucins are also found in normal cells and are not absolutely specific for cancer; c) mucins are extremely large and have many specific epitopes that can induce immunity; d) it is difficult to analyze the epitopes exposed in a tumor in individual cancer patients; e) side effects can be considerable and immune reactions against monoclonal antibodies prevent high dosage of antibodies; f) the glycosylation mucins within a tumor changes due to the aberrant expression and activities of glycosyltransferases, altering mucin antigenicity. To overcome these problems, a number of different mucin constructs have been developed as antigens, containing peptide and carbohydrate tumor antigens, with several different adjuvants and co-stimulating compounds, and various methods of immune stimulation.

Mucin vaccines based on MUC1

MUC1 is consistently overexpressed in cancer and has been a primary candidate for cancer vaccine design [67]. Vaccines are promising since spontaneous anti-MUC1 immune responses in cancer patients are associated with better prognosis or with a reduced lifetime risk of developing MUC1 positive cancers. A number of vaccine formulations based on MUC1 have been tested during the past 30 years in vitro using cancer cell lines, in mice and in patients with carcinomas. Most domains of MUC1 were used to prepare peptide or glycopeptide antigens for a vaccine. Important components of effective vaccines are adjuvants that induce strong immune responses of the innate and adaptive immune system. Thus, natural or modified mucin epitopes have been linked to KLH, bovine serum albumin

(BSA) or Tetanus toxoid derived from *Clostridium tetani*. Tetanus toxoid is a strong immune stimulant and elicits immune responses and high titers of IgG antibodies in mice [73]. The levels of cell surface-bound and circulating mucins, as well as their glycosylation patterns are variable and may be tumor- and individual-specific, making the choice of appropriate vaccine very difficult. Glycosylated MUC1 carrying Tn or sialyl-Tn antigens showed a significant immune stimulation and, when linked to a cell-penetrating peptide, delayed tumor growth in mice [102].

MUC1 glycopeptides linked to BSA were produced containing unnatural GalNAc β -Ser/Thr linkages as analogs of the Tn antigens and produced high antibody titers in mice [103]. In order to create new mucin glycopeptide epitopes and stable conformations of the O-glycans, the O-linkage of GalNAc to Ser/Thr was replaced by an unnatural S- or Se-linkage. The synthetic S- and Se-glycosides were conjugated to gold nanoparticles and bound to anti-MUC1 antibodies. Vaccination with this immunogen in mice resulted in a significant humoral immune response against cancer-MUC1 without adjuvants [69].

Conjugation of a MUC1 peptide antigen to a β -glucan polysaccharide, which serves both as a carrier and an immune activator in nanoparticles, facilitated the delivery of antigens leading to the activation of both innate and adaptive immunity [104]. Peptide- β -glucan conjugates can form uniform nanoparticles that facilitate the delivery of antigens. Beta-glucans also binds to multiple cell surface receptors and lectins stimulating immune processes such as T cell and B cell activation.

A successful anti-cancer vaccine was composed of MUC1 and a TLR7 agonist as adjuvant covalently conjugated to BSA. This adjuvant-protein-antigen [105] enhanced humoral and cellular immune responses in mice. The raised antibodies showed a potent anti-MUC1 response to human breast cancer cells MCF-7.

However, mucin-based vaccines have generally shown low responses in human patients. Therefore, further improvement has focused on the modifications of the mucin glycopeptide epitopes and choice of adjuvants [106]. Vaccine constructs range from synthetic mucin antigens conjugated to carrier proteins, encapsulations in liposomes or nanoparticles, or addition of T-helper epitopes to stimulate the induction of memory B-cells to permit long-term immunity. The combination of vaccine and additional drugs such as indomethacin can result in a significant reduction in tumor burden [107].

The bacteriophage Q β is a powerful virus-like particle for antigen delivery due to its highly organized three-dimensional structure and ability to display cancer antigens. A conjugate of Q β and MUC1 carrying the T antigen has shown success in vitro in killing of B16 melanoma cells expressing MUC1 after addition of complement. Furthermore, mice vaccinated with Q β -MUC1 glycopeptide conjugates were protected from

forming pulmonary metastases induced by B16-MUC1 melanoma cells [108].

In order to achieve strong tumor-specific immune responses, an antigenic epitope linear Gal (Gal α 1–3Gal) on MUC1 was produced. MUC1 was expressed in human pancreatic cancer cells transfected with α 1,3-Gal-transferase that synthesize the linear Gal epitope. Mice lacking the α 1,3-Gal-transferase were treated with pig tissue (expressing the epitope) to pre-immunize them, followed by B16F10 melanoma cells to induce tumors. The linear Gal-MUC1 vaccine successfully reduced tumor burden in these mice and significantly extended survival time [109]. Antibodies to the linear Gal epitope enhanced recognition and uptake of linear Gal-MUC1 by antigen presenting cells and thus improved the effectiveness of vaccination. Cancer stem cells are instrumental in proliferation of tumors and are often resistant to therapies. When tumors were induced with human pancreatic cancer cells PANC1 expressing the linear Gal epitope in mice, the immune responses were also directed against pancreatic cancer stem cells. Most of these stem cells express CD44, CD24 and epithelial-specific antigen. Stem cells were isolated with anti-CD44 antibodies and were shown to express MUC1 with the linear Gal epitope [109]. This suggests that antibodies against MUC1 with linear Gal epitope could eradicate stem cells. It remains to be shown if this strategy is applicable to human tumors.

MUC1 participates in the tumorigenicity and metastasis of colorectal cancer stem cells and has preferred epitopes for stem cell vaccination [110]. Stem cells from human colorectal adenocarcinoma cells SW620 express MUC1 and other markers such as the cell surface glycoprotein CD133 and aldehyde dehydrogenase. The injection of stem cell lysates into mice significantly reduced the growth of tumors induced by SW620 cells and increased NK killing activity. A comparison to lysates from SW620 cells after knockdown of MUC1 showed that the anti-tumor effect of the vaccine was dependent on the presence of MUC1. The vaccination did not show any toxicity.

A new peptide ligation technology is the diselenide-selenoester ligation (DSL) used for the preparation of self-advanting glycolipopeptide vaccines. Rapid ligation of MUC1 glyco- and lipopeptides is achieved via DSL together with a lipopeptide adjuvant. This vaccine construct elicited MUC1-specific antibody and cytotoxic T lymphocyte responses in mice. An additional helper T cell peptide epitope was able to boost the antibody response as well as cytokine production [111].

An interesting approach was taken in vaccine design by Glaffig et al. [112]. They utilized the Man receptor CD206, a C-type lectin, present on macrophages and dendritic cells to facilitate the uptake of vaccine consisting of MUC1 peptide carrying the Tn antigen and modified by Man residues. After vaccination of BALB/c mice, the Man-linked vaccine bound

to CD206 was taken up by cells and induced a high titer of IgG1 and IgG2 against MUC1.

A recombinant vaccine was produced with MUC1 linked to maltose-binding protein (MBP) and a human TLR-9 agonist as a DNA-based adjuvant [113]. Melanomas were induced in mice with MUC1-expressing B16 cells and T cell responses were examined after repeated injections of the vaccine. MUC1-specific responses included Th1 responses and cytotoxic lymphocyte killing activity with an increase in tumor infiltration of lymphocytes. Thus, a significant anti-tumor response was observed although additional injections of the vaccine decreased the numbers of immune stimulatory cells within tumors. This indicated that the timing of vaccine administration was crucial for the efficacy of vaccination.

Another covalent construct of seven VNTR peptides of MUC1 and MBP expressed in *E. coli* was used together with BCG as adjuvant to examine the effect on cell growth of MUC1-expressing BL16-induced melanomas in mice [114]. The combined vaccine induced Th1 helper cells and cytotoxic T-lymphocytes that resulted in decreased tumor growth. BCG was critical for enhancing the effect of MUC1-MBP.

Plant cells can naturally encapsulate antigens and a vaccination is possible by ingestion [115] of plant cells that prevent proteins from being degraded in the upper digestive tract. A fusion protein was constructed with the C-terminal peptide of HER2 and seven tandem repeat peptides of MUC1 with minimal N- and O-glycosylation. The gene encoding the antigen construct was transfected into hairy root culture. Mice orally immunized with the vaccine based on these cells showed increased humoral and cellular immunity with a considerable rise in serum IgG and IgA directed against MUC1 and HER2.

In a self-adjuvanting, highly effective anti-MUC1 vaccine, the N-termini of MUC1 peptides were conjugated to one or two palmitoyl chains (to form amphiphilic Pam-MUC1 or Pam2-MUC1) [116]. These lipid-tailed MUC1 antigens were assembled into liposomes containing Gal α -ceramide as an adjuvant and resulted in a significant immune response in mice. Vaccines containing other hydrophobic components as adjuvants were similarly effective.

L-Rhamnose (Rha) is an antigenic sugar that is foreign to humans, and anti-Rha antibodies have been found in humans. The priming of both CD4+ and CD8+ T cells toward MUC1 carrying Tn antigen was enhanced in mice that received human anti-L-Rha antibodies prior to vaccination with an L-Rha-modified MUC1-Tn cancer vaccine [117]. In order to exploit Rha as an adjuvant, a liposomal preparation of the TLR2 ligand Pam₃Cys (having 3 palmitoyl chains) linked to a Rha-containing glycopeptide from MUC1 carrying GalNAc (Tn) was evaluated as a vaccine in groups of C57BL/6 mice. Mice showed a strong humoral response. Some groups of mice were previously immunized to generate anti-Rha antibodies and exhibited particularly high cellular immunogenicity [118], indicating that Rha is a suitable adjuvant.

A tricomponent antitumor vaccine Pam3CysSer was used as an immune stimulator together with MUC1 glycopeptides, a tetanus toxin peptide as a T-helper epitope and fibroblast stimulating lipopeptide 1 (FSL-1) as adjuvant in covalent linkages [119]. B16 cell-induced tumors were grown in mice. The vaccine elicited both humoral and cellular immune responses, high IgG antibody levels and achieved a reduction in tumor size. Similarly, a vaccine was prepared with MUC1 glycopeptides (carrying GalNAc, the Tn antigen) linked to a TLR2 agonist (Pam3CysSK4, a peptide T-helper epitope derived from polio virus) or to a TLR9 agonist. Using a mouse model of mammary cancer, it was shown that vaccination resulted in potent antibody and cellular immune responses against tumor-associated glycopeptides [120, 121].

The SEA domain present in the extracellular moiety of MUC1 was chosen as an immunogen to raise monoclonal antibodies (mAbs) with high affinity for SEA [16]. The antibody reacted with MUC1 from tumors and cancer cells but only weakly with MUC1 from normal cells. A chimeric protein complex containing an IgG binding protein fused to exotoxin from *Pseudomonas aeruginosa* linked to an anti-SEA domain humanized antibody was tested in cancer cells in vitro and in immunodeficient nude and SCID mice in vivo. Tumors were induced in these mice with MUC1 positive pancreatic cancer cells Colo357. The chimeric protein complex showed high cytotoxicity and suppression of tumor volume.

Algae can be used to produce proteins and as delivery vehicles of vaccines. A multiepitope vaccine was produced with tumor-associated epitopes from HER2, MUC1, Wilm's tumor antigen and mammaglobin. The vaccine also contained the recombinant B subunit of enterotoxin from *Escherichia coli* as an immunogen that binds the glycosphingolipid GM1 and was expressed in algae. After injection of the vaccine, mice developed serum IgG antibodies that were shown to be directed against mouse breast cancer cells 4 T1 [122].

As DC are the major antigen-presenting cells, they can be primed with an antigen and then injected into the patient to enhance responses. MUC1 peptides can be used as antigen with various adjuvants. These primed cells were successful in reducing tumor load or growth [87]. Pre-activation of patient's T cells is another method to stimulate the immune response [123].

Clinical studies using MUC1 based vaccines

The initial studies of immune responses to mucin epitopes were conducted in cell lines, and then in mice before initiating clinical trials. Tecemotide vaccine contains a synthetic 25 amino acid, non-glycosylated MUC1 lipopeptide (BLP25), and the immunoadjuvant monophosphoryl lipid A in a liposomal preparation. Tecemotide injected into mice with lung cancer showed significant T cell responses against MUC1. Therefore, tecemotide was examined in lung cancer patients. Tecemotide with or without prior chemotherapy did not significantly

increase survival time in patients with unresectable stage III non-small-cell lung cancer, but the vaccine appeared to be successful in patients receiving chemo- and radiotherapy concurrent with the vaccine [88] (Table 1). The vaccine did not produce toxicity but did not show the expected effectiveness in reducing tumor burden. Further study in this population is warranted. Tecemotide vaccine was also tested in a clinical phase II study of patients with HER2 positive breast cancer [89], and the vaccine was found to be safe but did not improve tumor burden.

A vaccine based on vaccinia virus and MUC1 (TG4010) was examined in a phase II study of patients with metastatic renal clear-cell cancer with and without additional cytokines [90]. The side effects were minimal, and patients developing CD8⁺ T cell responses against MUC1 showed a longer survival. However, the study is still considered preliminary as many factors could influence the outcome.

MUC1 glycopeptides carrying Tn antigens were examined in the Rhesus macaque monkeys. The MUC1 (glyco) peptides were derived from Rhesus monkey mucin and were mixed with an adjuvant or with autologous DC. The vaccine showed high immune response when the Tn antigen was present [91]. Similarly, in a Phase I/II clinical trial with a limited number of patients with nonmetastatic castrate-resistant prostate cancer, the vaccine was found to be safe and able to induce significant T cell responses with a lesser increase in PSA levels (Table 1).

Theratope (from Biomira) is a vaccine comprising the Sialyl-Tn antigen linked to KLH protein that has shown success in patients with metastatic breast cancer to prevent relapse of the disease [92]. The initial injections contained oil emulsions with lipid A and cell wall fragments from *Mycobacterium phlei* as immune stimulants. An increase in survival of breast cancer patients was observed in a Phase II study with minimal toxicity of the vaccine. However, a follow up Phase III study could not confirm the survival results.

Another approach was a vaccine construct against multiple myeloma, consisting of the signal peptide of MUC1 which specifically addressed MUC1 but not its glycosylated or C terminal domains. This ImMucin vaccine was successful in Phase I/II human trials in eliciting antibodies as well as cell-mediated immunity against the construct, stabilizing the disease [93].

For adoptive immunotherapy, autologous DC were treated with full-length MUC1 mRNA, and autologous cytotoxic T-lymphocytes were generated in vitro. These cells together with the chemotherapeutic gemcitabine were injected into late-stage pancreatic cancer patients and led to a significantly higher survival rate [94]. Several other clinical trials have been conducted using MUC1 based vaccines with results that require further modification of vaccines and study designs [124] (Table 1).

Mucin vaccines based on MUC4

MUC4 is not expressed in normal pancreatic tissues at detectable levels but is present at high levels in pancreatic cancer

[23, 125, 126] and contributes to tumor aggressiveness. It has been shown that MUC4 expression modulates cell signaling, promotes tumorigenesis and metastasis and contributes to the resistance of pancreatic tumor cells to chemotherapy [126]. MUC4 expression progressively increases with the progression to pancreatic ductal adenocarcinoma, thus MUC4 can be used for diagnosis and is a target for cancer therapy. When the expression of MUC4 has been knocked down, proliferation, invasion, and metastasis of pancreatic cancer cells in vitro and in vivo are decreased [126, 127]. The appearance of anti-MUC4 antibodies and increased Th1 and Th2 cytokines in pancreatic cancer suggest strong immunogenicity of the mucin in cancer.

MUC4 is autocatalytically cleaved N-terminal to its vWF domain, forming the O-glycosylated domain MUC4 α and the membrane-anchored domain MUC4 β (Fig. 2). MUC4 has 3 EGF domains and interacts with a variety of proteins involved in tumor progression, including HER2, proteins of the Wnt pathway, galectin and integrins [128]. MUC4 modulates the interaction of tumor cells with their TME and contributes to the resistance of tumor cells to chemotherapy [129]. The proliferative action of MUC4 includes the inhibition of apoptotic pathways.

Several therapeutic avenues have been taken using MUC4 epitopes. Polyamphiphilic nanoparticles were synthesized containing encapsulated purified MUC4 β chain for their ability to activate dendritic cells and induce adaptive immunity. The MUC4 β protein can be released from the nanoparticles with minor loss of epitope. Immunization in mice with this nanovaccine led to MUC4 β -specific antibody responses [130, 131]. Immature dendritic cells when pulsed with MUC4 β -nanovaccine exhibited significant increase in the surface expression of MHC I and MHC II and costimulatory molecules (CD80 and CD86) and in the secretion of pro-inflammatory cytokines (interferon- γ , IL-6, and IL-12). The MUC4 β -nanovaccine elicited IgG2b and IgG1 anti-MUC4 β -antibodies in a ratio that suggested a predominantly Th1-like class switching.

Glycopeptides from the VNTR of MUC4 carrying T and sialyl-Tn antigens, conjugated to tetanus toxoid were used to vaccinate mice. The strong immune responses were specifically directed against MUC4. Analyses of antibody specificity by glycopeptide microarrays demonstrated that antibodies could distinguish between epitopes at specific glycosylation sites [132]. These studies suggest that MUC4 is an excellent candidate for an anti-tumor vaccine for pancreatic, lung, gastric, endometrial and other tumors expressing MUC4.

Conclusions

About 80% of patients with a variety of different tumors experience an increased expression of cell surface signaling

mucins MUC1 or MUC4 with abnormal glycosylation, making these mucins suitable targets for immunotherapy in cancer. Significant immune responses have been noted in in vitro and mouse models upon immunization with many different mucin constructs, often carrying cancer-associated O-glycans and a variety of adjuvants. However, there is a need for vaccines that induce effective anti-tumor responses and decrease tumor growth. The results of immunotherapy that suggest a positive outcome remain to be validated in clinical trials. The development of vaccines, antibody and T cell therapies that combine the tumor glycans (sialyl-)Tn or (sialyl-)T antigens and adjuvants for immunostimulation should be continued. Before choosing the appropriate vaccine, patient tumor antigens and serum mucins should be established to confirm aberrant expression, shedding, and glycosylation in order to direct the optimal immunotherapy with the highest probability of success. Mucin vaccination is a safe anti-tumor prevention method and can be considered adjuvant immunotherapy accompanied by additional anti-tumor strategies. Any alterations in cancer-associated antigens need to be monitored after treatment that may require therapy modification. Cell surface mucins MUC1 and MUC4 exhibit a more consistent expression phenotype in cancer compared to soluble mucins. With the exception of patients with high risk of cancer, mucin vaccines should probably not be a general preventative method due to the presence of a small number of cancer epitopes in normal tissues. Future studies on the glycosylation and antigenicity of cancer stem cells may lead to new approaches. However, efforts should continue to produce vaccine with targeted delivery to patient's tumor cells based on the high expression of glycosylated cancer-associated epitopes, together with support and effective stimulation of the patient's immune system.

Acknowledgements This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada (to I.B.).

Declarations

Conflicts of interests The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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