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# MUC1 in carcinoma-host interactions

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**Many carcinoma-associated markers are glycoconjugates whose expression undergoes temporal or spatial regulation. Mucin-1 (MUC1), discovered through monoclonal antibody technology, is a well-documented example of such a molecule and influences numerous pathophysiological behaviors, such as the invasion and metastasis of carcinoma cells. Levels of MUC1 expression in carcinomas correlate with the clinical stage of the cancer and inversely correlate with the survival prospects of patients. The MUC1 immune response is known to provide a protective host defense mechanism against cancer. The multiple functions of MUC1 in carcinoma-host interactions are believed to be dependent on the polymorphic nature of MUC1, particularly its glycosylation status.**

***Keywords:*** **MUC1 mucin, genetic polymorphism, O-glycosylation, tumor marker, cell adhesion, cancer immunotherapy**

## Introduction: MUC1 in cancer biology

The molecular characterization of MUC1 was initiated in many laboratories through initiatives to identify novel carcinoma-specific molecules using monoclonal antibodies. These antibodies were originally thought to bind to an epithelial surface marker highly expressed on breast, pancreatic, and other types of carcinoma cells [1,2]. Laboratories of Taylor-Papadimitriou, Hilkens, Wreschner, and Metzgar identified a highly glycosylated glycoprotein of high molecular weight as a carrier of putative carcinoma-specific antigenic epitopes which were recognized by specific monoclonal antibodies [3–6]. Reports by Finn's group proposed that the peripheral blood of breast and pancreatic carcinoma patients sometimes contains precursors of cytotoxic lymphocytes that are specific for a variety of carcinoma cells. The specificity was shown to be due to MUC1 [7–9]. The benefit of the MUC1-specific immune response was confirmed in a different way by Hilgers and co-workers, who showed that breast carcinoma patients with MUC1-specific antibodies in their sera had a better prognosis [10]. We became interested in the relationship between MUC1 and cancer biology through the results of a different trail. During studies on the metastatic phenotype of human colon carcinomas using cell lines as an experimental model, we found that levels of high molecular weight sialoglycoproteins on the cell surface positively correlated with metastatic potential [11]. The differential expression of these sialoglycoproteins was suggested not to be due to changes in cellular levels of glycosylation, but to differential levels of transcription of the glycoprotein gene. The high molecular weight glycoproteins were found to correspond to MUC1, and MUC1 expression in human colon carcinoma tissues showed that MUC1 levels were higher in advanced stage carcinomas; however, the different expression levels did not seem to be due to variations in mRNA levels [12]. Liver metastasis showed a higher content of MUC1 than the corresponding primary tumors when specimens from the same patients were compared [12]. A similar difference was observed for renal cell carcinomas, and there was a clear inverse correlation between MUC1 levels of renal carcinomas and survival of the patients [13].

Mucins, the major epithelial luminal surface glycoproteins, are characterized by their high molecular weight ( $>200$ kDa) and high content of carbohydrate side chains (50–90%). Most of these carbohydrate chains are linked to a core protein containing a tandem repeat domain through serine and threonine residues. To date, at least 12 distinct epithelial mucin core protein genes have been identified in humans by cDNA cloning. These have been designated MUC1 [3–6], MUC2 [14], MUC3 [15], MUC4 [16], MUC5AC [17], MUC5B [18], MUC6 [19], MUC7 [20], MUC8 [21], MUC9 [22], MUC11 [23], and MUC12 [23] according to the chronological order in which the genes were cloned. All of them contain tandem repeats with high contents of serine or threonine residues. The reason why MUC1 was the only

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mucin 'discovered' by many laboratories focusing on cancer biology/immunology still remains a mystery. Mucins can be subdivided into membrane-associated and secretory mucins, and these categories probably reflect their unique functions. For a while, MUC1 was thought to be the only transmembrane mucin. However, MUC3, MUC4 and MUC12 have now been confirmed by full length cDNA cloning to have transmembrane domains. It is also well known that MUC1 is cleaved at its extracellular site [24], a characteristic that it shares with MUC4 [25]. Furthermore, MUC1 and MUC4 lacking tandem repeat domains have been reported [6,26]. Thus, MUC1 is by no means unique and, leaving aside its particular range of tissue distribution, is highly ubiquitous among all epithelia except for the gastrointestinal tract [27]. MUC1 is known to regulate carcinoma cell behavior *in vitro* and *in vivo*. Carcinoma cells with increased levels of cell surface MUC1 are known to be less adhesive towards each other. They are also less sensitive to natural killer cells than MUC1 negative carcinoma cells. Soluble and cell surface MUC1 was reported to suppress T lymphocyte function. Potentially, these characteristics of MUC1 may be useful for the clinical assessment of cancer patients, as patients having tumors with a high content of MUC1 have a poorer prognosis than those with a low MUC1 content. The diverse biological functions of MUC1 probably reflect to a great extent its molecular diversity, which relies on the number and the sequence of tandem repeats (genetic polymorphism), variations in mRNA splicing (post-transcriptional variations), and patterns of glycosylation (post-translational modifications).

## Molecular diversity of MUC1

The tandem repeat of MUC1 comprises the sequence HGVTSAPDTRPAPGSTAPPA. All O-linked oligosaccharides are thought to link to this domain. The PDTR sequence forms a polyproline $\beta$-turn helix type of secondary structure [28]. The amino acid side chains radiate outward from an extended rod-like backbone. According to Jentoft [29], an extensively *O*-linked glycosylated polypeptide of 28 amino acids has a length of approximately 7 nm. Thus, MUC1 is thought to protrude at least 200 to 500 nm above the cell membrane. The molecular diversity of MUC1 is based on the number of tandem repeats, the variations in mRNA splicing, and patterns of glycosylation. Furthermore, the MUC1 gene is highly polymorphic due to its variable number of tandem repeats (from 25 to $>125$ repeats), which correspond to the heavily glycosylated domain [30]. This polymorphism was first described for Caucasians [30]. The protein products of polymorphic MUC1 genes are also polymorphic and detectable in urine [30]. However, we noticed that short MUC1 mucin alleles, which were seen only in $\sim$30% of Caucasian genes, predominate in Japanese populations (Figure 1). Mutant genes within the tandem repeat domain have recently been reported [31]. Genetic polymorphism in the number of tandem repeats might influence the behavior of malignant epithelial cells expressing this molecule as explained below.

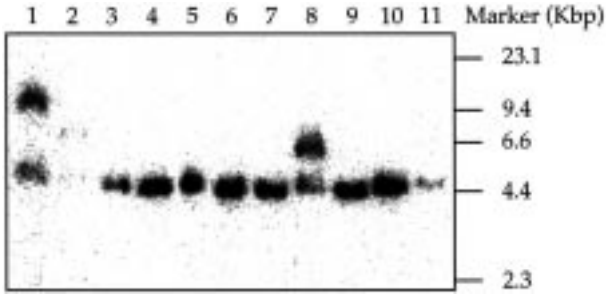


**Figure 1.** Southern blotting analysis of MUC1. Genomic DNA was prepared from HT29 cells, derived from a Caucasian, and peripheral blood mononuclear cells from healthy donors. The DNA was digested with Hinf I, separated electrophoretically in 0.7% agarose gels, transblotted onto nylon membrane and the membranes were probed with a partial sequence of the MUC1 gene, pMUC10 (Gendler SJ *et al.*, 1987, Proc. Nat. Acad. Sci. USA., 84: 6060–4). Lane 1: HT 29 cells, Lane 2–11: healthy Japanese donors. Two bands are observed in lanes 1, 2 and 8. Only one band is seen in 8 out of 10 Japanese individuals. The pattern indicates that there are less numbers of tandem repeats present in Japanese populations than in Caucasians and that the resulting mucin of Japanese populations is probably shorter than that of Caucasians, who are often heterozygous for two distinct sizes of MUC1.

The diverse glycosylation of MUC1 associated with malignant epithelia is believed to generate immunogenic epitopes useful for the detection and potential immunological eradication of malignant epithelial cells. Many monoclonal antibodies specific for MUC1 and cytotoxic lymphocytes obtained from pancreatic, mammary, and ovarian carcinoma patients were found to recognize the peptide sequences within the tandem repeat [32]. The sequence was proposed to be more accessible on malignant epithelial cells than on normal counterparts due to decreased glycosylation (Figure 2) [33]. It was shown by Lloyd and co-workers that the difference could be explained by a higher degree of *O*-glycan extension through the core 2 structure [34]. It has also been reported that GalNAc attachment to threonine residues within the most predominant peptide epitope of many anti-MUC1 peptide antibodies, APDTR, enhanced antibody reactivity [35]. Bhavanandan also conducted structural characterizations of MUC1 from normal urine and from laryngeal carcinoma cells, and concluded that glycan structures and the degree of glycosylation were very similar [36,37]. Endeavors to understand the 'unique' glycosylation of MUC1 on malignant epithelial cells has greatly contributed to our understanding of the regulation of mucin glycosylation. Many UDP-GalNAc-peptide *N*-acetylgalactosaminyltransferases were discovered and their specificity was investigated, revealing incredible fidelity in their substrate specificity [38–43]. The regulation of *O*-glycosylation of MUC1 and MUC2 was reviewed by us in a separate article [44] and by Hanisch [45].

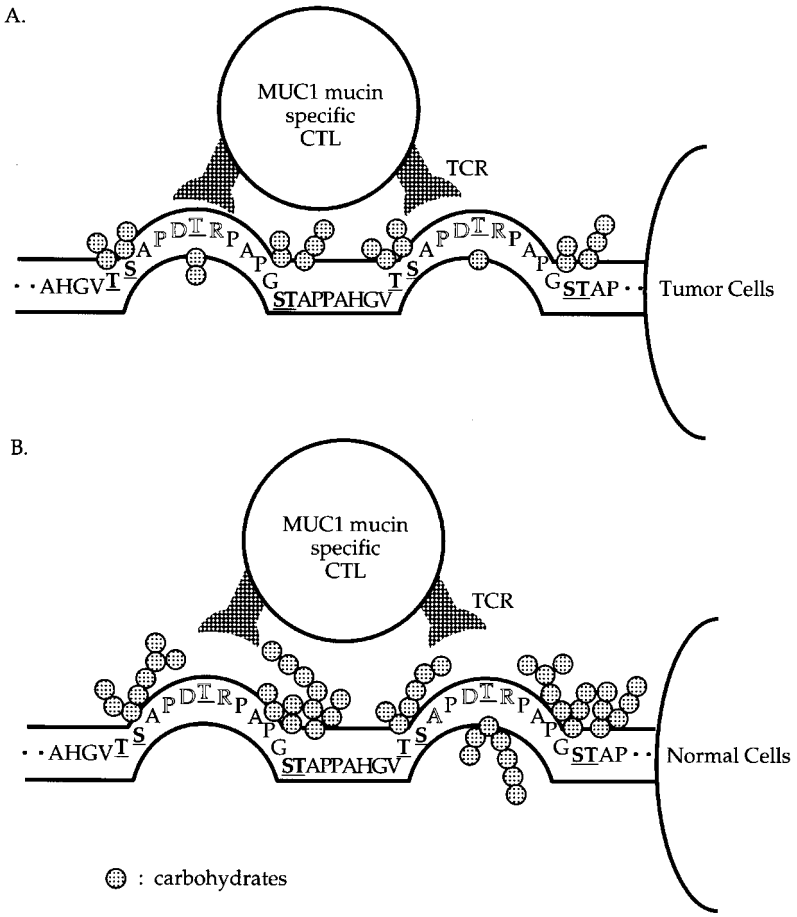


**Figure 2.** Model for MHC-unrestricted recognition of the MUC1 peptide epitope by MUC1 specific CTLs. The epitope PDTR within the tandem repeat is thought to be more accessible on malignant cells (A) than on normal cells (B) due to lower degree of extension of *O*-glycans.

## Clinical correlation

Immunohistochemical examinations of more than 50 specimens of surgically resected colorectal carcinomas indicated that MUC1 expression, detected by mAb HMFG-1, was higher in specimens derived from patients with advanced stage carcinoma [12]. In carcinomas of the ampulla of Vater, patients with tumors positive for MUC1 showed significantly poorer survival rates than those with MUC1 negative tumors [46]. In renal cell carcinomas, MUC1 levels were inversely correlated with the post surgical survival of patients (Figure 3) [13]. The difference in expression seemed to be due to differential transcription of the MUC1 gene and not to changes in glycosylation. In bladder carcinomas, the staining intensity with the anti-MUC1 core peptide mAb correlated with both the tumor grade and stage of tumor progression [47]. From similar clinical studies, it was suggested that MUC1 expressed by breast, ovarian, and pancreatic carcinomas enhanced the metastatic capacity of these tumors [48–50]. Interestingly, MUC1 has not been reported to correlate inversely with the aggressiveness of carcinoma cells. There are many clinical observations indicating correlation between the expression of

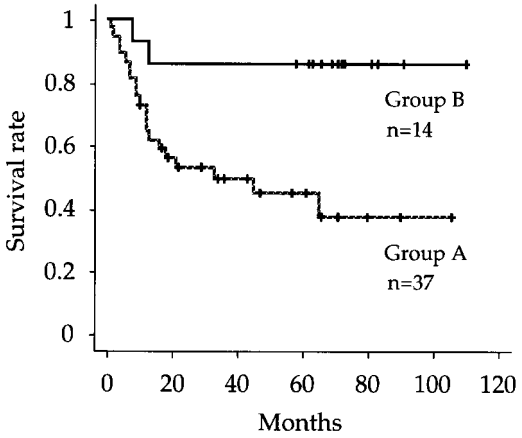


**Figure 3.** Relationship between survival and expression of MUC1 in renal cell carcinomas as determined by mAb MY.1E12 binding. The figure indicates Kaplan-Meier's survival curves. The patients were divided into two groups according to the intensity of mAb MY.1E12 staining. Group A had greater or equal to 5% of the cells stained, and Group B had less than 5% of the cells stained ($p = 0.009$ according to the log-rank test).

carcinoma-associated carbohydrate antigens, such as sialyl Lewis a, sialyl Lewis X, Tn and sialyl Tn and the clinical stage or prognosis. Although these carbohydrate antigens are likely to be associated with mucins, the core polypeptides presenting these carbohydrate epitopes remain obscure. It has been shown that patterns of glycosylation of MUC1 expressed in pancreatic carcinoma cells were distinct from those of other glycoproteins [51]. Hanski proposed that sialyl Lewis X was attached to MUC1 on colon carcinoma cells growing *in vitro*, whereas this epitope is carried by MUC2 on cells in clinical specimens. It remains to be assessed whether these observations are a common feature of all tumor types.

## MUC1 in cell–cell interactions

A major question is why do high levels of MUC1 expression correlate with the poor prognosis of patients having different carcinomas. MUC1 is a long and rigid glycoprotein that extrudes out of the cell membrane. Therefore, increased MUC1 expression is thought to alter interactions between tumor cells, and between tumor cells and host cells. As a result, the pathophysiological nature and immunological behavior of the tumor may be greatly modified by variations in MUC1 expression.

### Anti-adhesive property

In normal epithelial cells, MUC1 is expressed only on the apical (luminal) side of the cells. In carcinoma cells, however, this polarization is lost to some extent and over-expression of MUC1 on the whole cell membrane is believed to destabilize cell–cell adhesion and permit cancer cells to migrate and metastasize [52]. This tendency to lose homotypic cell adhesion was experimentally observed under the influence of a potent adhesion molecule, E-cadherin, as reported by Hilkens and co-workers [53]. They used double transfectant cells that expressed both MUC1 and E-cadherin to show that MUC1 could prevent intercellular adhesion mediated by E-cadherin [53]. According to their report, the length of the tandem repeats, but not the degree of sialylation, was the dominant factor that determined the inhibition of E-cadherin-mediated cell–cell interactions. Furthermore, MUC1 over-expression inhibits integrin-mediated cell adhesion to the extracellular matrix [54]. It was reported that MUC1 is related to the increased invasive ability of MUC1 transfected human gastric cancer cells [55]. This effect was abolished by treatment with an inhibitor of O-glycan extension, benzyl-α-GalNAc. This suggests that the extracellular domain may be involved in the enhancing effect of MUC1 on cell motility. However, it has not been clearly demonstrated that the anti-adhesive property of MUC1 correlates with the metastatic ability of tumor cells *in vivo*.

As stated above, sialyl Lewis X and sialyl Lewis a epitopes are found in MUC1. MUC1 molecules with these epitopes are likely to function as ligands for carbohydrate-binding adhesion molecules such as selectins [56–61]. Furthermore, under-glycosylated MUC1 has been shown to function as a ligand for the intracellular adhesion molecule 1 (ICAM-1) [62]. Thus, association or dissociation of cells expressing MUC1 may depend on the glycosylation pattern of the expressed MUC1 and on the nature of the molecules on the surface of cells that interact with MUC1-expressing cells.

### Influence of MUC1 on tumor growth *in vivo*

As stated above, clinical observations have demonstrated that high levels of MUC1 expression are indicative of a more aggressive stage in tumor progression and poor survival. However, there have been few attempts to elucidate the function of MUC1 on the basis of clinical data. We demonstrated an inverse correlation between MUC1 expression in renal cell carcinomas and patient survival [13]. To assess whether MUC1 expression is causally related to malignant tumor behavior, MUC1 cDNA was stably transfected into a renal carcinoma cell line SN12C that expressed trace levels of MUC1 [63]. Contrary to our expectations, there was no correlation between MUC1 expression and *in vitro* growth or motility. *In vivo* growth of the MUC1 transfectants at the site of orthotopic transplantation in nude mice was slower than mock transfectant cells. MUC1 may play an important role in tumor growth. The *in vivo* growth rate of breast tumors induced by polyoma middle T antigen was found to be significantly slower in mice deficient in Muc-1, a mouse homologue, than in wild type mice [64]. The tumor growth of MUC1 cDNA transfected gastric cancer cells in nude mice was increased compared to that of mock transfected cells [55]. However, transfection and expression of MUC1 cDNA did not always promote growth of tumor cells. For example, MUC1 cDNA transfected colon carcinoma cells grew slower than mock transfected cells because of the anti-adhesive property of the MUC1 transfected cells [65]. The effect of MUC1 on tumor growth may be attributed to other unknown molecules that associate with MUC1.

### Interactions with cells in the immune system

MUC1 is known to suppress immune functions through a variety of mechanisms. Cells coated with MUC1 adhere less efficiently to natural killer (NK) cells, lymphokine activated killer (LAK) cells and cytotoxic T cells (CTLs), and are resistant to killing by these cells [66–68]. MUC1 secreted from a colon carcinoma cell line has been reported to inhibit target cell lysis by NK cells [67]. Secreted MUC1 carrying sialyl Lewis X and sialyl Lewis a epitopes inhibits leukocyte adhesion to cells expressing E-selectin [69]. The inhibitory effects of soluble MUC1, found in the ascitic fluids of human breast cancer patients, may also be mediated by its interaction with ICAM-1 [62].

MUC1 was shown to have immunesuppressive effects on T cells. Agrawal and co-workers reported that MUC1 purified from ascitic fluids of ovarian cancer patients and synthetic

peptides containing MUC1 tandem repeats suppressed T cell proliferative responses, but could not induce apoptosis [70]. This suppression of T cell responses was reversed by the addition of exogenous IL-2. These findings, however, are still controversial. Paul et al. suggested that certain other molecules that can associate with MUC1, such as amino sugars or other mucins, might be responsible for the anti-proliferative effects [71]. The polymorphic nature of MUC1, which may be crucial for the immunosuppressive effects, has not been considered from this perspective.

### MUC1 in signal transduction

A high degree of conservation of the cytoplasmic domain of MUC1 during evolution is suggested by cross-mammalian species [72]. Tyrosine residues in the cytoplasmic domain are phosphorylated and tyrosine phosphorylated MUC1 directly interacts with the Src homology 2 domain of the adapter protein Grb2 [73,74]. The cytoplasmic domain of MUC1 directly interacts with $\beta$-catenin, depending on the state of cell adhesion [75]. The expression of the MUC1/Y isoform, which is an alternative splicing variant devoid of tandem repeats, was shown to increase tumorigenicity [76]. The secreted form of MUC1 interacts with MUC1/Y, inducing the phosphorylation of the MUC1/Y protein and producing profound affects on cell morphology [77]. Therefore, the cytoplasmic domain of MUC1 may play a role in intracellular signaling.

## MUC1 as a target of cancer immunotherapy

### MUC1-specific cytotoxic lymphocytes and antibodies

MUC1-specific CTLs have been isolated from draining lymph nodes of pancreatic and breast cancer patients, ascitic fluids of ovarian cancer patients, and peripheral blood mononuclear cells of a multiple myeloma patient [7–9,78]. These MUC1 specific CTLs express T cell receptor (TCR) $\alpha/\beta$, CD3, and CD8, and recognize MUC1 directly in a major histocompatibility complex (MHC)-unrestrictive manner. Direct recognition of the MUC1 peptide epitope by the TCR in the absence of presentation by the MHC was shown to induce the same early activation events that followed conventional MHC-restricted recognition [79]. The highly multivalent epitopes of tandem repeats in a single MUC1 molecule cross-link the TCRs of MUC1-specific CTLs. The anti-MUC1 monoclonal antibody SM-3, which recognizes the tumor-associated mucin core peptide PDTRP [80] within tandem repeats, was shown to block this CTL activity, indicating that the epitope of MUC1-specific CTLs was an underglycosylated MUC1 core peptide within the tandem repeats. NMR data revealed that the PDTR sequence formed a knob-like structure that protruded away from the long-axis of MUC1 [81]. Due to the shorter carbohydrate chains of mucins in some carcinomas, such as breast and ovarian carcinoma, the accessibility of the repetitive sequences of the protein core is increased in tumor tissue. The reactivity of MUC1-specific CTL against MUC1 transfectant cells was enhanced by blockade of *O*-glycosylation [82,83]. Whether the aberrantly glycosylated MUC1 expressed by breast and other carcinoma cells carries 'nonself' T-cell epitopes remains a subject for further investigation. MHC class I-restricted cellular immune responses to MUC1 were also observed in multiparous women and in murine models [84,85].

A humoral anti-MUC1 reaction was also detected in patients with ovarian or mammary carcinomas [86,87]. Furthermore, early breast cancer patients with naturally occurring MUC1 antibodies have a better chance of survival [10]. MUC1 antibodies may also function as important effectors. A surprising finding was that MUC1 is also expressed on non-epithelial cells and on cells in the immune system, such as activated T cells and dendritic cells [88,89]. Closer examination of the subcellular localization and status of glycosylation of MUC1 may provide keys to clarify this paradoxical situation.

### MUC1-based cancer vaccine

A variety of ways to immunize with a soluble synthetic MUC1 peptide derived from the tandem repeat region were tested for their efficacy to protect against tumor challenges in murine tumor models. Ding et al. showed that KLH-conjugated 16-mer synthetic peptides elicited high IgG responses [90]. Apostolopoulos et al. showed that mice immunized with soluble MUC1 or a 20-mer synthetic peptide showed potent anti-MUC1 antibody responses but little CTL activity and even less protection against tumors [91]. They immunized mice with conjugates of mannan-MUC1 fusion protein, which produced MHC-restricted $CD8^+$CTLs. In contrast, immunized patients produce high antibody titers with poor CTL responses to MUC1 [92]. Goydos et al. vaccinated adenocarcinoma patients with 105 amino acid synthetic peptides. Immune responses could be detected in some, but not all patients [93]. It has previously been shown that antibodies against the Gal$\alpha$1-3Gal epitope, which are normally present in humans but not in mice, cross-react with MUC1 peptides [94]. In humans but not in mice, these crossreactive anti-$\alpha$Gal antibodies were deviating the immune response to antibody production [95]. Thus, it seems crucial to develop methods to break tolerance to MUC1 in humans.

Expression profiles of MUC1 in transgenic mice expressing human MUC1 from its own promoter are similar to those seen in humans [96]. MUC1 transgenic mice are tolerant to stimulation by MUC1 at the level of the T cell [97]. Therefore, MUC1 transgenic mice are expected to provide a useful model to investigate the mechanisms regulating immunological tolerance to tumor antigens and should facilitate the investigation of anti-MUC1 immunotherapy. MUC1 transgenic mice rejected tumor cells modified to secrete IL-12, but not IL-2, IL-4, or IFN-$\gamma$ [98]. Mannan-MUC1 fusion protein alone could generate an immune response to MUC1 and the response was enhanced by combining the mannan-MUC1

fusion protein with a recombinant vaccinia virus expressing IL-12 [99,100]. Gong and coworkers induced antitumor immunity by immunizing with fused hybridomas of dendritic cells (DCs) and carcinoma cells in transgenic mice expressing human MUC1 [101,102].

To design tumor vaccines, the glycosylation status of MUC1 should be considered. Antigen presentation by professional antigen presenting cells (APCs) is very important for the induction of T cell responses specific for MUC1. Hiltbold et al. reported that the glycosylated secreted form MUC1 from ascites of patients with breast or pancreatic tumors is not processed by DCs and does not elicit MHC-class II-restricted T helper responses *in vitro* [103]. In contrast, a synthetic MUC1 peptide of 100 amino acids is naturally processed and presented by MHC class II molecules on DCs. The glycosylated form of MUC1 is readily taken up by DCs, but is not transported to late endosomes or MHC class II compartments for processing and binding to MHC class II [104]. Furthermore, they examined the ability of DCs to present the three glycosylated forms (glycosylated, underglycosylated and nonglycosylated) of MUC1 by MHC class I [105]. The efficiency of processing and the resulting strength of CTL activity were inversely correlated with the degree of glycosylation. Therefore, the synthetic nonglycosylated peptide seems to be the most suitable for making vaccines.

On the other hand, it was reported that glycopeptides or glycosylated MUC1 are better than nonglycosylated peptides for making vaccines. In breast cancer patients, natural antibodies to MUC1 reacted more strongly with GalNAc peptides, while this reactivity was significantly reduced by nonglycosylated peptide vaccination [106]. CTLs induced by the lymph node lymphocytes of colorectal carcinoma patients, which preferentially killed MUC1-expressing target cells, recognized a carbohydrate epitope, possibly T-antigen on underglycosylated MUC1 [107]. Furthermore, HLA-A0201 restricted CTLs were induced by immunization with the human milk fat globule membrane antigen (HMFG) linked to mannan in HLA-A0201/Kb × MUC1 double transgenic mice [108]. The epitopes of CTLs induced by mannan-HMFG were present throughout the whole MUC1 molecule. These observations indicate that HMFG, glycosylated MUC1 can be processed and presented on APCs. The optimal site and structure of glycosylation required for the induction of the immune responses remain to be elucidated.

## Conclusions

MUC1 is not only a promising candidate for cancer immunotherapy but also a molecule affecting the malignant behavior of carcinoma cells. The correlation between the aggressive clinical behavior of certain carcinomas, such as breast, ovary, colon, and renal carcinoma, and the expression of MUC1 in clinical specimens is obvious. However, it has not been clear whether molecular functions of MUC1 are causally related to the behavior of tumor cells *in vivo*. Furthermore, the involvement of MUC1 in interactions between tumor cells and the host immune system is complex. MUC1 is known to protect cancer cells from the immune system, but it also serves as a target of immune surveillance. MUC1 was recently found to be expressed on hematopoietic cells and cells in the immune system. Thus, understanding the role of MUC1 in immunity is expected to become a more complicated issue in the near future. The molecular diversity of MUC1, including polymorphism, splicing variants, and glycosylation, may potentially provide the key to solve the mystery of MUC1 action in cancer biology and immunology.

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