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MINI-REVIEW

Mucin glycoproteins in neoplasia

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Mucins are high molecular weight glycoproteins that are heavily glycosylated with many oligosaccharide side chains linked O-glycosidically to the protein backbone. With the recent application of molecular biological methods, the structures of apomucins and regulation of mucin genes are beginning to be understood. At least nine human mucin genes have been identified to date. Although a complete protein sequence is known for only three human mucins (MUC1, MUC2, and MUC7), common motifs have been identified in many mucins. The pattern of tissue and cell-specific expression of these mucin genes are emerging, suggesting a distinct role for each member of this diverse mucin gene family. In epithelial cancers, many of the phenotypic markers for pre-malignant and malignant cells have been found on the carbohydrate and peptide moieties of mucin glycoproteins. The expression of carbohydrate antigens appears to be due to modification of peripheral carbohydrate structures and the exposure of inner core region carbohydrates. The expression of some of the sialylated carbohydrate antigens appears to correlate with poor prognosis and increased metastatic potential in some cancers. The exposure of peptide backbone structures of mucin glycoproteins in malignancies appears to be due to abnormal glycosylation during biosynthesis. Dysregulation of tissue and cell-specific expression of mucin genes also occurs in epithelial cancers. At present, the role of mucin glycoproteins in various stages of epithelial cell carcinogenesis (including the pre-neoplastic state and metastasis), in cancer diagnosis and immunotherapy is under investigation.

Keywords: mucin glycoproteins, mucin genes, carbohydrate antigens, cancer, metastasis

Introduction

It has long been demonstrated that quantitative and qualitative alterations in mucin glycoproteins occur in epithelial cancer cells using histochemical and biochemical methods [1–6]. Although the precise mechanism of these alterations is still not well understood, considerable progress has been made during the past decade in our understanding of the changes that occur in malignant transformation of epithelial cells. This is due to considerable advances in monoclonal antibody and lectin technology, sensitive analytical methods for studying small amounts of glycoprotein samples, molecular cloning, sequencing and expression methods as well as cell biological methods. In this review, the biochemical properties of mucin glycoproteins, their synthesis and the current state of our knowledge on human mucin genes will be briefly discussed. Then the mucin glycoprotein changes that occur in epithelial mucosal cells during carcinogenesis progressing from pre-malignant to malignant and metastatic states will be described. And finally, the possible functional significance of these changes and diagnostic and therapeutic potential will be discussed. Although several different types of epithelial cancers will be mentioned, mucin glycoproteins in gastrointestinal neoplasia such as colorectal and pancreatic cancer will be discussed in more detail.

Biochemical properties of mucin glycoproteins

Mucin glycoproteins consist of a protein backbone with many carbohydrate side chains of varying lengths, sequence, compositions and anomeric linkages. They have a very large molecular weight (400 to >1000 kDa), many 'O-glycosidically' linked carbohydrate side chains which

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may constitute 50–85% of the total molecular weight, a high content of serine, threonine and proline in the protein backbone structure, and a buoyant density much higher than non-glycosylated proteins (1.35–1.50 $g\,cm^{-3}$) due to their high carbohydrate content [7–9]. Mucins may be broadly classified into two forms: membrane associated or secreted. The secreted forms can perhaps be further subdivided by their propensity to form a gel and the nature of the gel formed.

Mucin genes and protein backbone structure (apomucins)

Heterogeneity of mucin species in particular organs has long been suspected. However, little was known about the structure of apomucins until recently. This is mainly due to the heavy glycosylation of mucins which makes biochemical characterization difficult. Most procedures used to deglycosylate mucins result in extensive degradation of the polypeptide backbone. With the recent application of molecular biological methods, however, the structures of apomucins are beginning to be understood. To date, at least nine human mucin genes have been identified (Table 1) [10–24]. Although only MUC1, MUC2 and MUC7 human mucin cDNAs have been fully sequenced, available data from mammalian [25–28] and amphibian mucins [29, 30] indicate that mucins share certain structural features. For example, a predominant feature of most, if not all mucins is the central region consisting of repeat peptide sequences flanked on either side by non-repetitive domains. The repeat unit of each mucin gene differs in the amino acid sequences and the lengths of these repeats, but they all have a high content of threonine and/or serine, potential O-glycosylation sites. The repeat sequences are repeated in tandem along the length of the molecule and thus are called tandem repeat regions.

An example of membrane-bound mucin is MUC1, shown in Fig. 1. This mucin has been previously

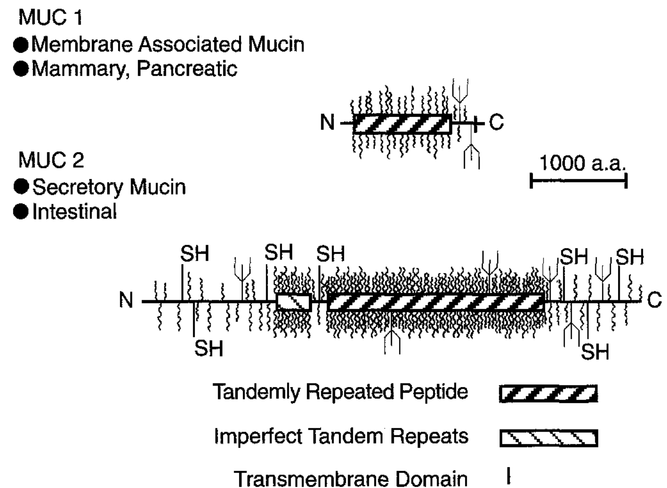

Figure 1. Schematic drawing of MUC1 and MUC2 structure. MUC1 is a membrane mucin that is thought to protrude from the cell surface with a rigid but hydrophilic structure. It contains relatively short amino and carboxyl terminus domains. The transmembrane domain is found near the carboxyl terminus. MUC2 is a secretory mucin. It has two internal repetitive domains that are rich in potential O-glycosylation sites (Ser and Thr residues). As described in Fig. 2, sequences with homology to vWF flank the internal glycosylated domains and comprise the majority of the amino and carboxyl termini.

Table 1. Characteristics of human mucins. The sequence of the repeat peptide, tissues exhibiting high expression, and chromosomal location are given.

Mucin type	*Repeat peptide sequence*	*High level expression*	*Chromosomal location*
MUC1	PDTRPAPGSTAPPAHGVTSA	Breast, Pancreas	1q21
MUC2	PTTTPITTTTTVTPTPTPTGTQT	Colon, S.I., T.B.	11p15
MUC3	HSTPSFTSSITTTETTS	Colon, S.I., G.B.	7q22
MUC4	TSSASTGHATPLPVTD	T.B., Colon	3q29
MUC5AC	TTSTTSAP	T.B., Stomach	11p15
MUC5B[a]	SSTPGTAHTLTVLTTTATTPTATGSTATP	T.B.	11p15
MUC6	SPFSSTGPMTATSFQTTTTYPTPSHPQTTLPTHVPPFSTSLVTPSTGT-VITPTHAQMATSASIHSTPTGTIPPPTTLKATGSTHTAPPMTPTTSGTS-QAHSSFSTAKTSTSLHSHTSSTHHPEVTPTSTTTITPNPTSTGTSTP-VAHTTSATSSRLPTPFTTHSPPTGS	Stomach, G.B.	11p15
MUC7	TTAAPPTPSATTPAPPSSSAPPG	Salivary	4
MUC8[b]	TSCPRPLQEGTPGSRAAHALSRRGHRVHELPTSSPGGDTGF	T.B.	

[a]The DNA encoding this tandem repeat array exhibits insertions and deletions thus altering the reading frame. The most common amino acid sequence encoded is shown.
[b]The encoding DNA consists of a 41 bp array with occasional deletions, thus regular reading frame shifts are observed with MUC8. This results in different portions of the basic repeat unit being alternatively expressed.
[c]S.I., small intestine.
T.B., tracheobronchus.
G.B., gallbladder

designated as PUM, PEM (polymorphic epithelial mucin), MAM-6 and DF-3 [10–13, 31–33]. MUC1 is highly expressed in lactating mammary glands and is a major glycoprotein component of the milk fat globule membrane. This mucin is often highly expressed in certain adenocarcinomas, particularly of the breast and pancreas. By convention, this mucin has been named MUC1 and subsequent mucins are numbered sequentially in order of their discovery. The *MUC1* gene is highly polymorphic due to different alleles with variable numbers of tandem repeats, and has been mapped to human chromosome 1q21–24. The protein encoded by *MUC1* is 120–300 kDa in size in the apomucin form and 300–600 kDa in the glycosylated form. It has a relatively large extracellular domain extending above a short transmembrane sequence (31 amino acids) with a cytoplasmic domain of 69 amino acids in the carboxyl terminus which may interact with cytoskeletal components. The extracellular domain contains a large central tandem repeat region consisting of 20–80 peptide units (depending on the allele) of 20 amino acids each containing 25% serine and threonine (potential O-glycosylation sites) extending rod-like approximately 200–500 Å above the cell surface. MUC1 is typically localized to the apical membrane of epithelial cells and may shield epithelial surface molecules such as receptors and antigens in certain pathological conditions [33–35]. Another membrane associated mucin-like molecule, leukosialin (CD43) present on leukocytes, has been reported to be synthesized and released into the medium by a colon cancer cell line [36].

The secretory mucins constitute the major component of mucus gels that cover the epithelial surfaces of the gastrointestinal, respiratory and reproductive organs. Eight distinct human secretory mucins have been described to date [6]. In the human intestine, at least two forms of secreted mucins are present in abundance, MUC2 and MUC3 [15, 16, 22, 37–41]. Sequence analysis suggests that these mucins have substantially different structures. MUC2, the most studied secreted form of human mucin, is a very large protein and consists of approximately 5100 amino acids in the most common allelic form (Fig. 1). The dominant structural feature of the protein is a central core of approximately 100 tandem repeats of 23 amino acids each. This central core region contains a very high density of threonine residues (potential O-glycosylation sites) and is flanked on either side by unique sequences that also contain a fairly high density of potential O-glycosylation sites. Upstream, there is another repetitive sequence in the amino-terminus portion of the molecule. It contains imperfect tandem repeats ranging from 7 to 40 amino acids with a very high density of both threonine and serine. As is the case with MUC1 protein, there are potential N-glycosylation sites particularly in the non-repetitive region. The unique sequences that flank the tandem repeat region are very rich in cysteine, which may be involved in joining mucin monomers together to form large molecules. This model is consistent with the long thread-like molecules observed in electron microscopy of mucins.

In addition to two highly glycosylated tandem repeat domains, MUC2 has amino-terminal signal peptide sequences followed by four D domains (three in the amino terminus and one in the carboxyl terminus) that are cysteine-rich regions with a high degree of sequence similarity to four D-domains of pre-pro vWF [39] (Fig. 2). In addition to the D-domains, there is considerable sequence similarity between MUC2 and pre-pro vWF in other regions of the molecule. By analogy to pre-pro vWF which is well characterized with regard to the formation of large polymers (>12 000 kDa) via intermolecular disulfide bond formation, these regions may be important in the processing and polymerization of MUC2. Moreover, genetic lesions in this region of the molecule could result in a mucin unable to polymerize.

MUC3 is a second major intestinal mucin [16]. Only the partial sequence of this mucin is known. The *MUC3* gene is located on chromosome 7q22 [40] and encodes a repetitive peptide of 17 amino acids containing seven threonine, five serine and one proline residue. The carboxy terminal region down stream of the tandem repeat region consists of a 'mucin-like' domain rich in threonine, serine and proline and a cysteine rich domain with an epidermal growth factor-like structural motif.

The molecular functions of MUC2 and MUC3 may be substantially different. Because of its cysteine-rich composition and similarity to vWF, MUC2 may form a

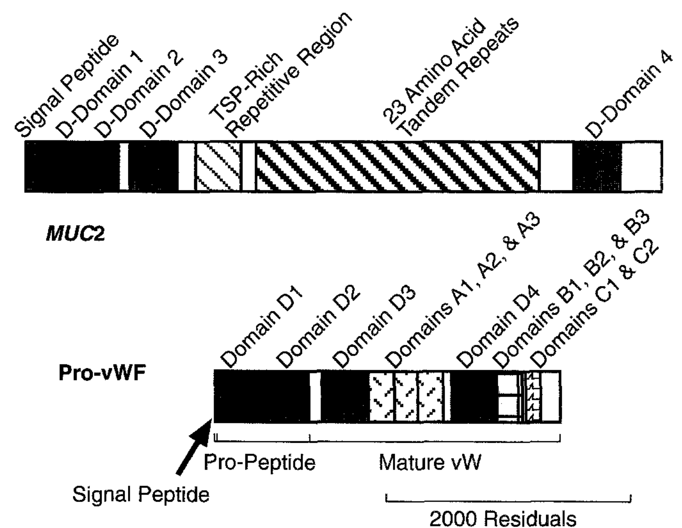

Figure 2. Common domain structure of MUC2 and Pre-Pro vWF. In addition to its internal glycosylated domains, MUC2 also contains four domains with significant similarity to the D-domains of vWF. Other portions of the molecule exhibit significant similarity of vWF as well, including a cysteine rich region near the carboxyl terminus that may be involved in dimer formation.

highly polymerized structure that functions as a physical barrier. Although the complete sequence of *MUC3* is not yet known, it clearly has a very different structure and may even be incapable of polymerization. The presence of an EGF-like carboxy terminal domain suggests a possible role in ligand/receptor interactions involving pathogens on intestinal mucosal cell surfaces. For example, this interaction may facilitate the wound healing process since the addition of mucin glycoproteins alone or with human intestinal trefoil factor caused attenuation of the damage to intestinal epithelial cell monolayer integrity [42, 43].

Recently, two major human gastric mucin genes have been characterized, *MUC5AC* (*MUC5*) and *MUC6* [17–20, 22, 41]. Both mucin genes are highly expressed in the stomach but *MUC5* is also highly expressed in the airway and *MUC6* in the gallbladder and small intestine. Tandem repeat units of *MUC5* consist of eight amino acid residues while that of *MUC6* consists of 169 amino acid residues. 3′ cysteine-rich domains are present in both types of mucin. The carboxyl terminus of MUC5 has sequence similarity to pre-pro vWF. By analogy to MUC2 and pre-pro-vWF, MUC5 may protect against HCl and peptic digestion. MUC6 mucin may be a soluble mucin functioning to protect glands locally from digestion or may be secreted to bind bacteria to be eliminated from the stomach [42].

In addition to the relatively organ specific pattern of expression, mucin genes are also expressed in a cell type specific manner. For example, *in situ* hybridization and immunohistochemical studies indicate that MUC2 is expressed in goblet cells while MUC3 is expressed in both goblet and absorptive cells of the intestine [37, 44]. MUC5 is expressed predominantly in surface mucous cells in both fundus and antrum of the stomach while MUC6 is expressed in the neck mucous cells of fundus and antral or pyloric gland cells [44–46]. It is of interest that four mucin genes, *MUC5AC, MUC5B, MUC6* and *MUC2* are very closely linked and located in the chromosome 11p15 gene cluster. The significance of this interesting clustering of mucin genes is unclear.

Carbohydrate side chains of mucin

The majority of the carbohydrate side chains of mucin glycoproteins are attached to the protein backbone through O-glycosidic linkages between *N*-acetylgalactosamine (GalNAc) and serine/threonine residues. Although it was long thought that mucin glycoproteins contain only O-glycosidically linked oligosaccharides, recent studies indicate that a small number of N-glycosidically linked carbohydrates (between *N*-acetylglucosamine and asparagine of the protein backbone) may also be present in mucin glycoproteins [47, 48]. Five different sugars are commonly found in O-glycosidic mucin glycoproteins. These are *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), *N*-acetylneuraminic acid (NeuAc), galactose (Gal) and fucose (Fuc). Carbohydrate side chains are often branched, with 1 to >20 sugars per chain. They may be negatively charged, owing primarily to NeuAc and ester sulfate on Gal or GlcNAc. Up to a third of the total amino acid residues may be linked to carbohydrate side chains, making the protein backbone resistant to proteolysis. O-linked oligosaccharide side chains may be arbitrarily divided into three regions: inner core, backbone, and periphery. The sugar or sugars that are attached to the GalNAc that is linked to the protein backbone compose the core region carbohydrates. Eight different types of core region carbohydrates have been identified [7, 49–51] as follows: Core 1, Galβ1-3 GalNAc-R; Core 2, GlcNAcβ1-6[Galβ1-3]GalNAc-R; Core 3, GlcNAcβ1-3 GalNAc-R; Core 4, [GlcNAcβ1-6] GlcNAcβ1-3 GalNAc-R; Core 5, GalNAcα1-3 GalNAc-R; Core 6, GlcNAcβ1-6 GalNAc-R; Core 7, GalNAcα1-6 GalNAc-R; and Core 8, Galα1-3 GalNAc-R. Although the precise relative proportions of core sugar structures of mucins from different organs are not known, core 3 structures appear to be the predominant one in normal human colon [52, 53], while core types 1 and 2 are found in normal gastric mucin [54, 55].

In the normal human adult gastrointestinal mucosa in man, the core structures are usually elongated by the major basic backbone structure consisting of a type 1 backbone chain (Galβ1-3 GlcNAcβ1-3Galβ-R). This backbone structure may be replaced with a type 2 (lactosamine) chain (Galβ1-4 GlcNAcβ1-3Galβ-R), and less frequently with a type 3 chain (Galβ1-3 GalNAcα1-3Galβ1-R) and a type 4 chain (Galβ1-3 GalNAcβ1-3Galβ1-R) [4]. The type 2 chain may be repeated in the same oligosaccharide side chain to form a poly *N*-acetyllactosamine backbone, but the type 1 chain is usually not repeated. The type 1 and type 2 chain backbone structures can also be branched in a β1-6 configuration using Gal as branch point. The backbone chains are terminated or terminally branched by α-linked sugars such as NeuAc, fucose, *N*-acetylgalactosamine, *N*-acetylglucosamine or galactose in the peripheral region and terminal sulfate may be present. All three regions of the oligosaccharide side chains provide specific recognition sites for carbohydrate-specific antibodies and can serve as tumor markers recognized by monoclonal antibodies and lectins. Except for the core region carbohydrates which are unique to mucin glycoproteins, most of the backbone and peripheral region carbohydrates may be found in both the O- and N-glycosidic glycoproteins and in glycolipids [4].

Alteration of mucin glycoproteins in cancer

Previous histochemical studies indicated that both qualitative and quantitative changes in mucins occur in cancer

[1, 3]. In the case of colon cancer, these include a decrease in mucin content, a decrease in sulfomucin, an increase in sialomucin and a decrease in O-acetylated sialic acid [1, 3]. Recent biochemical studies on mucin glycoproteins isolated from normal and cancerous colons indicate a reduction in carbohydrate content, and fewer and shorter oligosaccharide chains in colonic cancer mucin [2, 56–58]. Although further extensive analyses of mucin oligosaccharides of normal and cancerous colonic mucosa are necessary, the predominance of the core 1 structure in cancerous colonic mucin is evident. Analysis of mucin isolated from human rectal cancer [59, 60] and human colon cancer cell lines [61] showed core types 1, 3 and 5 and core types 1, 2 and 4 respectively. This contrasts with normal colonic mucins which apparently contain primarily core 3 structures [52, 53]. Thus, there appears to be a major difference between core structures of normal and cancerous colonic mucin.

With the recent availability of monoclonal antibodies of defined specificities, structural alterations in mucin glycoproteins in epithelial cancer cells are beginning to be defined. Three groups of mucin glycoprotein tumor markers with different specificities are shown (Table 2). These tumor markers include those with carbohydrate epitopes, those specific for peptide moieties of mucin glycoproteins, and those for which the epitope specificities have not been determined.

Carbohydrate antigens

Cancer associated changes in the carbohydrate moieties of mucin may be broadly classified into four categories: those arising from incomplete synthesis, increased levels of expression over that found in normal tissues, modification of existing structures, and inappropriate or incompatible expression of antigens (Table 3). These changes could occur in the peripheral, backbone and inner core regions of oligosaccharide side chains. Incomplete synthesis is illustrated by the appearance in adenocarcinomas of core region carbohydrates Tn, T (Thomsen-Friedenreich) and sialyl Tn antigens (Table 4) which are normally masked by additional sugar residues in normal tissues [62–68]. Tn and T antigens have been reported to be expressed in greater than 90% of primary adenocarcinomas and their metastasis [62]. Sialyl Tn is also considered to be a 'pan-carcinoma' antigen. This antigen has been reported to be expressed in adenocarcinomas of the colon (94%), breast (84%), lung (non-small cell, 96%), and ovary (100%) and in most pancreatic, gastric and esophageal cancers examined [65]. Tn, T and sialyl Tn antigens are not detectable in mucosa of normal colon but are expressed in colon cancers (Tn 81%, T 71%, sialyl Tn 89%) [5, 67, 68]. The T antigen is preferentially expressed by moderately well differentiated and well differentiated adenocarcinomas while Tn antigens are also present in poorly differentiated adenocarcinoma and colloid cancer of the colon [67]. Furthermore, Tn, T and sialyl Tn antigens are expressed in over 50% of adenomatous polyps and the expression of these antigens is correlated with the malignant potential of polyps (size, histological type and degree of dysplasia) [68]. These findings indicate that these antigens may serve as useful premalignant and malignant markers. Using MAbs, T and sialyl T antigens were shown to be present in high molecular weight mucin glycoproteins isolated from human carcinoma cell lines [69, 70].

The examples of modification of existing structures include sialylation of type 1 backbone chains without or with fucosylation which are CA-50 antigen [71, 72], sialyl lacto-*N*-tetraose and sialyl Le^a antigen (NeuAcα2, 3Galβ1,3[Fucα1,4]GlcNAc) [72], also known as CA19-9 [72] and GICA antigen [73, 74] (Table 4). SPan-1 MAb recognizes the epitope on both CA50 and sialyl Le^a antigens [75, 76], while DU-PAN2 MAb recognizes only the CA50 epitope [77]. These antigens are found on glycolipids and mucin and non-mucin glycoproteins of a variety of adenocarcinomas. Interestingly, these antigens are released into the circulation or secreted into pancreatic secretions mainly on mucin glycoproteins [76, 78]. Although serum sialyl 19-9, CA50 and SPan-1 defined antigens have a relatively high degree of specificity for pancreatic cancer, these antigens are expressed in both normal and cancerous pancreas [75–77, 79–81]. In contrast with normal colon, sialyl Le^a expression is increased in fetal colon, hyperplastic and adenomatous polyps, and in most colorectal cancers regardless of location or differentiation [79]. Sialyl Le^a expression does not correlate with stage, DNA aneuploidy or prognosis in colorectal carcinoma patients [75]. Most endometrial, gastric, and pancreatic carcinomas

Table 2. Mucin glycoprotein tumor markers of epithelial cancers.

Carbohydrate epitopes
Peripheral and backbone region carbohydrate changes
A, B, H, Le^a, Le^b
Sialyl Le^a (CA19-9), sialyl type 1 chain (CA50), SPan-1
Sialyl Le^x, extended Le^x, polymeric Le^x
Extended Le^y
Type 2 polyactosamine (i)
Core region carbohydrate changes
T, Tn, sialyl Tn
Peptide epitopes
Apomucins
MUC1–MUC6
Undetermined epitopes
M1 antigen, crypt cell antigens
SIMA, LIMA, NCC-CO-450 antigen
DU-PAN2, YPan-1, CA-125

Le, Lewis antigen.

Table 3. Cancer-associated changes in the carbohydrate moiety of mucin glycoproteins.

Incomplete synthesis
- Deletion of normally expressed antigens (e.g. deletion of A, B and Le^b antigens)
- Blocked synthesis with accumulation or exposure of precursors (e.g. expression of T, Tn and sialyl Tn)

Increased level of expression over that found in normal tissues (e.g. short chain Le^x and Le^y)

Modification of existing structures
- Extension/elongation of type 2 carbohydrate chain (e.g. i antigen)
- Increased sialylation and fucosylation of type 1 and/or type 2 carbohydrate chains [e.g. sialyl Le^a (19-9), sialyl Le^x, extended Le^x and Le^y, polymeric Le^x]
- Decreased O-acetylation of sialic acid (expression of sialyl Tn, sialyl Le^x

Inappropriate or incompatible expression of blood group antigens
- Inappropriate: (re-expression of ABH and Le^b antigens in colon cancers in distal colon where these antigens are not expressed)
- Incompatible: (expression of incompatible A, B, H, Le^a and Le^b antigens)

Le^b, Lewis antigen.

express focal sialyl Le^a immunoreactivity, whereas only 5% of breast adenocarcinomas are positive [73, 79, 80].

Numerous alterations of the type 2 backbone chains have been described in adenocarcinomas of the gastrointestinal tract, breast, and lung (Table 3). Type 2 chains synthesized as a simple, repeating, unbranched structure (poly *N*-acetyl lactosamine backbone structure i.e. i antigen), are highly expressed in colon and hepatocellular carcinomas and in large cell and squamous cell carcinomas of the lung, and are absent or weakly expressed in corresponding normal tissues [81]. In the normal colon, most type 2 chains are branched by β1,6GlcNAc transferase leading to synthesis of I antigens. Fucosylated and/or sialylated type 2 chains are also preferentially expressed by carcinomas compared with normal epithelium. The distribution of these antigens in colonic and pancreatic disease has been described in detail [82–85]. Short chain Le^x determinants are expressed in the normal colon with decreasing proximal to distal gradient, and are absent in normal pancreas. Expression of this antigen is enhanced in colorectal and particularly pancreatic cancer. Extended Le^x type antigens (Table 4) recognized by monoclonal antibodies FH4, and FH6 are rarely expressed in normal colon and pancreas or chronic pancreatitis specimens. In contrast, these antigens are expressed in the majority of adenomatous polyps and colon and pancreatic cancers. The di- and trifucosylated Le^x structures are recognized by the MAb FH4, while sialylated Le^x structures are recognized by the MAb FH6 [82]. Expression of these antigens in adenomatous polyps correlates with criteria indicative of a greater potential for malignant transformation (increasing size, villous histology, and dysplasia) [84]. The finding of FH6 immunoreactivity in 60% of hyperplastic colonic polyps which do not have malignant potential indicates that these antigens may also become expressed in mucosa with abnormal growth characteristics which are not considered to be premalignant [83].

The extended and trifucosylated type 2 chain (Le^y type) antigens are weakly expressed in normal colon (proximal site only) but highly expressed in the majority of adenomas with increased risk for malignant transformation and in colon cancers [84]. These epitopes are absent in hyperplastic polyps which do not have malignant potential. However, these extended or polyfucosylated Le^y antigens do not differ between normal,

Table 4. Structures of carbohydrate antigens.

Antigen	*Structure*
Tn	GalNAcα-Thr/Ser
Sialyl-Tn	NeuAcα2,6 GalNAcα-Thr/Ser
T	Galβ1,3 GalNAcα-Thr/Ser
i	Galβ1,4 GlcNAcβ1,3Galβ-R
Sialyl Le^a	NeuAcα2,3 Galβ1,3(Fucα1,4)GlcNAcβ-R
Le^x	Galβ1,4(Fucα1,3)GlcNAcβ-R
Difucosyl Le^x	Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4(Fucα1,3)GlcNAcβ-R
Sialyl Le^x	NeuAcα2,3 Galβ1,4(Fucα1,3)GlcNAcβ-R
Sialyl Le^x (extended)	NeuAcα2,3 Galβ1,4(Fucα1,3)GlcNAcβ-1,3Galβ1,4(Fucα1,3) GlcNAcβ-R
Le^y (short)	Fucα1,2Galβ1,4(Fucα1,3)GlcNAcβ-R
Le^y (extended)	Fucα1,2Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4GlcNAcβ-R
Le^y (polymeric)	Fucα1,2Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4(Fucα1,3) GlcNAcβ-R

benign or malignant disease in the pancreas [85]. These antigens are considered to be oncodevelopmental since they are expressed in neoplastic and fetal tissues but rarely in normal adult tissues.

In the human embryo and fetus, blood group antigens A, B, H, Le^a and Le^b are expressed throughout the colon [86, 87]. After birth, however, ABH and Le^b antigens are no longer expressed in the distal normal colon. These antigens are re-expressed in 90% of all cases of cancer in the distal colon. Furthermore, in 60–80% of cases of cancer of both the proximal and the distal colon, blood group antigens incompatible with the host's blood type are expressed [88]. About one-third of cases of adenomatous polyps re-expressed A, B and H antigens or expressed incompatible blood group A or B antigens. Deletion of A, B and H blood group antigens in pancreatic cancers was found to occur in 33% of cases; and incompatible expression of B antigen only was found in 13% of cases [80, 89]. Thus, re-expression and/or incompatible expression of blood group A or B antigens appears to be a pre-malignant or malignant phenomenon.

Mucin-related antigens with undetermined epitopes

Some of these mucin-associated antigens are not fully characterized chemically. These include Crypt Cell Antigen 90, M1 [91], SIMA, LIMA [92], Cora antigen [93], NCC-CO-450 antigen [94] YPan-1 [76] and CA-125 [95]. These antigens are generally strongly expressed by a variety of adenocarcinomas relative to corresponding normal tissues, and are frequently secreted into the circulation (Table 2).

Biochemical mechanisms of altered carbohydrate antigens

In considering the biochemical mechanisms responsible for the altered expression of carbohydrate antigens in cancer cells, the following possibilities may be considered: dysregulation of glycosyltransferases resulting in changes in enzyme activity and/or specificity for specific substrates, altered availability of nucleotide sugar substrates or de novo expression of a novel glycosyltransferase leading to altered pathways of glycosylation. It is now well established that glycosyltransferase genes are expressed transcriptionally in a tissue and cell specific manner [96]. For example, Galα2,6-sialyltransferase mRNA varies up to 100-fold in various rat tissues as does its enzyme activity. Genomic sequencing of Galα2,6-sialyltransferase indicates that in this family of sialyltransferases multiple transcripts are produced via a combination of alternative splicing and alternate promoter use which is regulated in a tissue-specific fashion [97]. However, it is not known whether other glycosyltransferase genes have similar organizational and regulatory structure. The Galα2,6-sialyltransferase [98] and Galβ1-3GalNAcα2,3 sialyltransferase [99] activities are increased in colon cancer.

The biosynthesis of mucin associated O-glycan structures and related antigens in cancer cells appears to be subject to complex control mechanisms. Although it is difficult to generalize, recent studies indicate that a significant reduction in the activity of GalNAcβ1, 3GlcNAc transferase synthesizing core 3 appears to be in part responsible for the increase in Tn and T (core 1) antigens in colon cancer tissues [99–101] (Fig. 3). The biochemical basis for the increased expression of sialyl Tn antigen in cancer cells is unclear. However, the available data indicate that it may be in part due to the fact that in normal tissues particularly in colon, O-acetylation of sialic acid predominates masking the antigenic epitopes of sialyl Tn, while in neoplastic tissues there is a marked reduction in O-acetylation of sialic acid [102–104]. A similar explanation may account for the increased expression of sialyl Le^x and sialyl Le^a antigens in colon cancer [104]. This raises the possibility that there may be a difference in the level of O-acetyltransferase activity between normal and cancerous tissues.

Regarding altered backbone structure, the accumulation of i antigen, an extended unbranched type 2 chain appears to be due to an increase in β1,3GlcNAc transferase activity in addition to a loss of branching in colonic adenocarcinoma [100, 105]. As for modifications of existing structures resulting in the expression of extended sialyl Le^x antigen in cancer cells, expression of α1,3 fucosyltransferases capable of modifying acceptors containing α2,3 sialic acid substituted lactosaminoglycans appears to be the critical step. A similar process may apply to the expression of sialyl Le^a where the expression of α1,4 fucosyltransferase capable of modifying acceptors containing α2,3 sialic acid substituted type 1 chains appears to be an important step. Thus, the two critical steps in the synthesis of sialyl Le^x and sialyl Le^a antigens are the α2,3 sialylation of type 2 and type 1 chain followed by α1,3 or α1,4 fucosylation of sialylated precursors, respectively. Several different α2,3 sialyltransferase and α1,3 fucosyltransferases have been cloned and their acceptor specificities analysed. At least one sialyltransferase (ST3N) and one fucosyltransferase (FUT3) are thought to be able to generate both sialylated type 1 and type 2 chains and both subterminal Fucα1,3 and Fucα1,4 linkages thus producing either sialyl Le^x or sialyl Le^a epitopes respectively, depending on the type of carbohydrate backbone structures available [106–109]. However, when α3/4 fucosyl transferase activity was compared in normal and cancerous colonic tissues, no apparent difference was noted [99].

In the case of A, B, H, and Le^b and Le^a blood group antigens, glycosyltransferase activities responsible for A

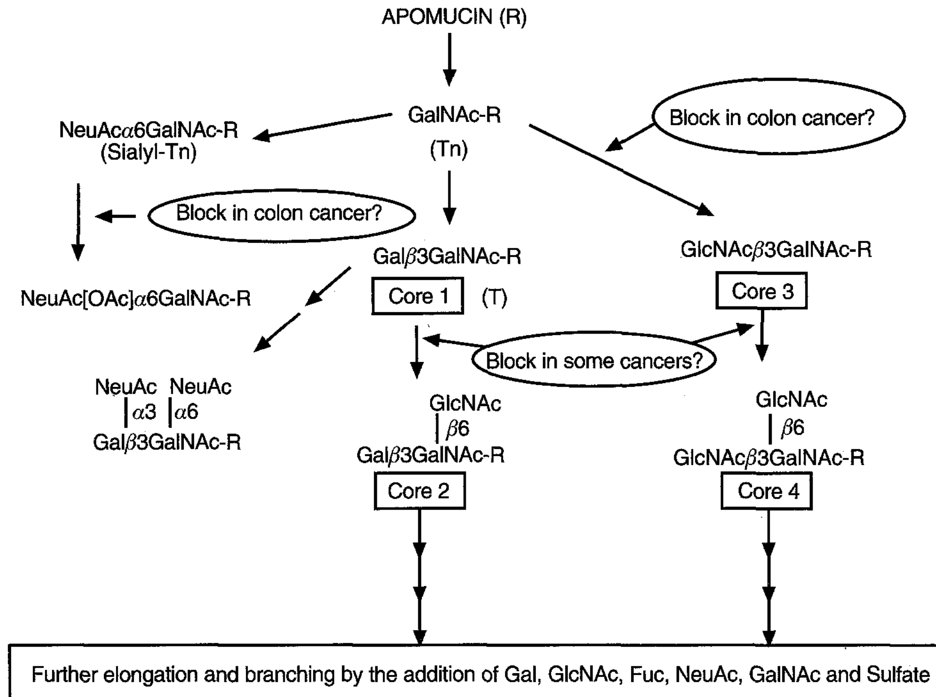

Figure 3. Biosynthesis of some core region carbohydrates of mucin glycoproteins. Gal, galactose; Fuc, fucose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; NeuAc[OAc], O-acetyl, N-acetylneuraminic acid.

and B determinants were reduced in colon cancer tissues compared to normal tissues, while those involved in the synthesis of non-blood group determinant carbohydrate structures remained at a similar level [56, 110]. Recently, when the presence of immunoreactive blood group antigens was compared with the activities of glycosyltransferase and glycosidase enzymes in normal colon and colon cancers, the activities of their corresponding glycosyltransferase enzymes were the same in proximal and distal normal colonic specimens, although blood group A, B, H, and Le^b antigens were not found in human adult distal colon [111]. Cancer tissues had generally lower A and B glycosyltransferase activities but similar H glycosyltransferase activity compared with paired normal mucosa. No difference in glycosidase activities were observed. However, A and B glycosyltransferase activities showed no direct relationship to the level of expression of A, B, and H antigens in cancer and paired normal mucosa. Similar findings were made in a study using colon cancer cell lines, and weak incompatible blood group A and B immunoreactivity was observed in two cell lines respectively [112].

The *A, B* and *O* alleles of the ABO blood group system are very similar. Although the full genomic structure is not known, *A* and *B* alleles differ by only 4 amino acids. The *O* allele differs by a single nucleotide deletion at nucleotide position 261, resulting in a shift in the reading frame giving rise to a truncated protein with no catalytic activity [113]. Recently, when 31 cases of gastric tumors of phenotype O were examined for the incompatible expression of blood type A and A-gene-defined glycosyltransferase, three cases were positive for the presence of both A antigen and A-gene defined enzyme. From these data it would appear that the incompatible blood group antigen in cancer may be synthesized by an active transferase protein derived from O gene by additional mutations (deletion/insertion) or alternative splicing [114]. These data indicate that although the presence of specific glycosyltransferase activity is necessary, it does not account entirely for the specific blood group antigen expression and that the altered availability of precursor substances (via diversion into alternate synthetic pathway) and the altered regulation of blood group gene specified enzymes (alternative splicing, mutations) may be important factors in the altered expression of blood group antigens in neoplasia.

Altered mucin gene expression in cancer

Recent studies indicate that the levels and the patterns of mucin mRNA and protein are frequently altered in adenocarcinomas compared to normal tissues. Alterations include increased expression, decreased or lost expression and aberrant expression. Studies using antibodies that recognize the tandem repeat sequences of mucin protein backbone structure often demonstrate increased immuno-

reactive mucin proteins in epithelial cancers compared with normal tissues, most likely due to incomplete glycosylation and unmasking and exposure of peptide epitopes [115–119]. With respect to MUC1, increased mucin peptide immunoreactivity generally correlates with increased mRNA in breast and other carcinomas [119, 120]. This has been attributed in part to increased *MUC1* gene copy number in one allele. Loss of one *MUC1* allele occurred in 29% of the tumor DNA among the tumor specimens that were informative (64%), while a gain of an allele or an additional copy of an existing allele has also been reported [119, 120]. Analysis of oligosaccharide side chains revealed that they were shorter in breast cancer cells than on the normally processed mucins, further supporting the theory that incomplete glycosylation contributes to increased exposure of peptide epitopes [121, 122]. The enzymatic basis for these changes in several breast cancer cell lines was shown to be an increase in core 1 α2,3-sialyltransferase expression and the loss of core 2 synthesis by β1,6GlcNAc transferase [123]. Although MUC1, 2 and 3 mucin peptide immunoreactivity is increased in premalignant polyps and cancer of the colon, the levels of mRNA (MUC1, 2 and 3) are decreased or unchanged in moderate to well-differentiated colon cancers and polyps [116, 124]. In contrast, MUC2 and MUC3 mRNA levels were increased in colloid colon cancer compared with normal tissue. Gastric cancers of all histological subtypes demonstrated increased MUC5 and MUC6 mRNA peptide immunoreactivity but decreased levels of mRNA expression [45, 125].

Furthermore, with malignant transformation the tissue and cell-specific expression of mucin genes becomes dysregulated [42]. For example, while normal pancreas mainly expressed MUC1 mucin with weak to undetectable expression of MUC2, MUC3, MUC4 and MUC5 at the mRNA and apomucin levels, pancreatic cancer tissues and cells showed increased expression of MUC2, MUC4, and MUC5 at the mRNA and apomucin levels in addition to MUC1 mRNA [126, 127]. In addition, different histological types of the tumors of the same organ may express different mucin types. In the pancreas, the MUC1 apomucin but not MUC2 apomucin is expressed in invasive ductal carcinoma while MUC2, but not MUC1 apomucin is expressed in intraductual papillary tumors with much better prognosis [117]. Similarly, mucinous carcinoma of the breast expressed both MUC1 and MUC2 apomucins while invasive ductal carcinoma of the breast expressed only MUC1 apomucin but not MUC2 [128]. The levels of mRNA and apomucin expression for MUC1, MUC5 and MUC6 are also low to barely detectable in normal colon but have recently been shown to be increased in premalignant type of adenomatous polyps and colon cancer [129]. In cholangiocarcinoma, non-invasive bile duct crypt adenomas with favorable prognosis generally expressed MUC2 apomucin while most invasive non-papillary cholangiocarcinomas with poor prognosis expressed MUC1 but not MUC2 apomucins [130]. When the intrahepatic duct cells were examined in fetal and adult livers, switching of MUC1 apomucin expression before birth to that of MUC3 was observed after birth [131]. Thus, the expression of MUC1 in invasive non-papillary cholangiocarcinoma represents oncodevelopmental phenomena.

Recent studies indicate that a colon cancer cell line HT29 which normally expresses *MUC2* and *MUC3* genes and only a trace level of the *MUC5* gene, upregulates MUC5 and mRNA at the transcription level in HT29-MTX cells selected by adaptation/resistance to methatrexate [132, 133]. This change is associated with significantly increased expression of gastric mucin and apomucin antigens in HT29-MTX cells indicating a shift of predominantly colonic type mucins to the gastric type. Clearly, the mechanisms involved in the regulation of cell and tissue specific expression of mucin genes in normal and malignant cells should be elucidated.

Mucin antigens in cancer progression and metastasis

Altered cell surface glycoproteins of cancer cells have been implicated in tumor progression to metastasis. For example, sialylated cell surface glycoproteins have been reported to be associated with enhanced metastatic potential in a variety of malignant cell types in experimental animals [134–136]. Colorectal cancer cells with increased metastatic potential have been reported to express altered lectin reactivities and altered expression of mucin glycoprotein associated carbohydrate antigens [138, 139]. These include reduced peanut agglutinin reactivity and increased expression of sialomucin, sialyl Tn and sialyl extended Le^x antigens. When the sialyl difucosyl Le^x antigen level was assayed in the tissue lysates of primary and metastatic human colorectal cancer specimens using antibody inhibition methods, it was higher in metastasis compared with primary tumors and lower in early stage primary tumors compared with more invasive or later stage primary tumors [140]. Immunoblot analysis indicated that this antigenic activity was associated with high molecular weight mucin-like glycoproteins in these specimens. Furthermore, inhibition of glycoprotein glycosylation by competitive inhibitors of sialyltransferase [141] or by aryl α-GalNAc [142, 143], a competitive inhibitor of GalNAcβ1,3Gal transferase and GalNAcβ1,3GlcNAc transferase, or the N-glycosylation inhibitor tunicamycin [144] results in diminished metastatic activity of cancer cells.

The mechanism by which sialylation alters metastatic activities of cancer cells appears to be multifactorial. Sialylation of mucin and other glycoconjugates may affect growth regulation, cellular or cell-substratum adhesion and invasion [145–150]. Sialylated tumor

associated antigens have recently been shown to be ligands for binding to selectins. Selectins are a family of cell adhesion molecules that share a common structural motif consisting of an amino terminal lectin-like domain [151]. Three main types of selectins, E, L and P are expressed on the cell surfaces of endothelial cells (E and P) leukocytes (L) and platelets (P). The ligands for the lectin-like domain of selectins include sialyl Le^x, sialyl Le^a, sulfated Le^x and sulfated Le^a antigenic epitopes. These structures are also frequently expressed on malignant cells and have been reported to be involved in selectin-mediated adhesion of cancer cells to endothelial cells and platelets, a prelude to subsequent colonization in the metastatic sites [152–155]. Recent studies suggeset that L- and P-selectins interact primarily with mucin-type ligands on colon cancer cells, while E-selectin can recognize ligands on both mucin and non-mucin type glycoproteins [156]. In addition, highly mucinous human colon cancer cell lines expressing both sialyl Le^x, sialyl Le^a and sulfated epitopes showed greater metastatic behavior in nude mouse models of human colon cancer metastasis [157]. This property could be significantly inhibited by treatment of the cells with Benzyl-αGalNAc, an inhibitor of mucin glycoprotein glycosylation concomitant with marked reduction in the expression of these antigenic epitopes by these cells.

Membrane-associated mucin glycoproteins have a rigid rod-like conformation that extends far above the plasma membrane. Studies on the effect of deglycosylation suggests that carbohydrates play an important role in the three dimensional structure of mucins due to hydrophilicity and anionic charge. Removal of sialic acids from mucin oligosaccharides reduces the extended conformation, while complete removal of oligosaccharides causes mucin to collapse like denatured globular proteins [158]. The overexpression of cell surface mucins may shield cell surface antigens or receptors from their ligands. Recently, MUC1 has also been demonstrated to have antiadhesion properties due to its highly sialylated extended and rigid rod-like structure [34, 35]. For example, transfection of MUC1 mucin cDNAs into human melanoma or fibroblast cell lines results in reduced cellular aggregation and reduced tumor growth and incidence in nude mice [159]. This suggests that MUC1 mucin may block normal cellular aggregation sites. The tandem repeat peptide of MUC1 apomucin was also expressed to a much higher extent in metastasis compared with primary colorectal tumors [160].

The expression of sialyl Tn and sialyl Le^x antigens in primary colorectal carcinomas has also been reported to be an important variable for predicting patient survival (disease-free and overall) [161, 162]. These observations strongly indicate the importance of carbohydrate dependent cellular behavior during carcinogenesis, tumor progression and metastasis.

Clinical application

No mucin glycoprotein antigenic markers have yet been shown to be sensitive or specific enough to be clinically useful as diagnostic serological markers for epithelial cancers. Although high levels of mucin glycoprotein antigens such as CA19-9, CA50, SPan-1 and DuPan-2 are observed in the serum of patients with pancreatic cancer and are relatively specific and sensitive for pancreatic cancer (about 80%), the serum assays for these antigens are used mainly to follow the course of the patients after surgical resection and/or chemotherapy [163–165]. CA15-3 is also elevated in the blood of some patients with cancers of breast, ovary and pancreas. This assay employs two MAbs directed to epitopes on the MUC1 apomucin [166]. However, it lacks the sensitivity and specificity to be clinically useful. At present, little is known about the mechanisms or factors leading to the high levels of these antigens in the serum of patients with cancer.

Monoclonal antibodies against mucin-related antigens may also be used for targeting radioisotopes (^{131}I, ^{111}In, ^{131}Tc) for imaging and therapy or for targeting cytotoxic drugs. Much of the work in this field has been carried out with MAb B72.3 directed to sialyl Tn and Tn epitopes [167–171]. Recently, other MAbs directed to epitopes on mucin glycoproteins, PAM4 [172] and Nd2 [79] have also been used particularly for pancreatic cancer. ^{111}In-labeled Nd2 MAb was found to detect relatively small pancreatic cancer in 67% of the cases [173]. Furthermore ^{131}labeled Nd2 MAb suppressed the growth of pancreatic cancer xenografts in nude mice [174] Nds MAb conjugated to Adriamycin injected either i.v. or intratumorally into athymic nude mice suppressed growth of pancreatic cancer xenograft tumors while free Adriamycin did not [175].

A form of passive immunotherapy is the administration of previously activated lymphocytes that attack tumor cells, e.g. adoptive transfer. Autologous tumor cells, allogeneic pancreatic cancer cell lines and autologous B cells that have been immortalized by infection with Epstein-Barr virus and transfected with MUC1 cDNA have been used to stimulate lymphocytes [176, 177]. These cells act as both stimulator and target cells for cytotoxic T lymphocytes (CTL). CTL cell lines developed from pancreatic patients inhibit tumor formation in nude mice when injected along with pancreatic tumor cells [178]. Cytotoxic T cells derived from pancreatic [179], breast [176] and ovarian [180] cancer patients recognize cell surface MUC1 tandem repeat peptide sequences. This recognition is not major histocompatibility complex-restricted presumably due to the presence of multiple well exposed, poorly glycosylated tandem repeat peptides which facilitate cross-linking of T cell receptors on mucin-specific T cells and the rod-like

conformation of MUC1 molecule on cancer cells, which may mask the histocompatibility complex [177, 180, 181]. Furthermore, cytotoxic T cells readily recognize target cells which are transfected with MUC1 cDNA encoding only two 20 amino acid tandem repeat sequences [182]. These data indicate that differences in glycosylation are mainly responsible for tumor associated mucin antigen expression and T cell recognition rather than differences in the mucin protein itself. This also indicates that a patient can mount an immune response against tumor-associated mucin glycoprotein components. However, stimulation of cytotoxic T cells by tandem repeat sequences of other mucins such as MUC2 and MUC3 have not been examined to date. In addition, it has been observed that cell surface carbohydrates containing α2,6 linked NeuAc may be important in a post-binding event in natural killer cell mediated lysis [183]. Recently, two monoclonal antibodies directed to Le^y-related, cell surface antigens of cancer cells (including colonic cancer cells) which exhibit a high tumor specificity for gastrointestinal and other epithelial cancers have been described. Both are internalized by antigen-positive cells and one monoclonal antibody has a direct cytotoxic effect on antigen positive tumor cells [184].

Stimulation of anti-tumor immune responses can also be accomplished by immunization with specific carbohydrate and peptide portions of mucin glycoproteins [4, 185]. Carbohydrate antigenic determinants with tumor specificity such as Tn [186, 187], T [187, 188] and sialyl-Tn [189, 190], are being tested. A synthetic tumor-associated conjugate consisting of synthetic T antigen coupled to carrier proteins was developed for active specific immunotherapy of a murine mammary carcinoma and was found to be useful in increasing the survival time of immunized mice compared with untreated ones [187]. Sialyl Tn is currently in Phase II clinical trials [191]. Cytokines modify the magnitude and specificity of the immune response to these antigens [192]. The peptide portion of MUC1 mucins [191, 193] and antiidiotype antibodies that mimic the structure of the MUC1 polypeptide [194] can also elicit immune responses. Human B cells from tumor draining lymph nodes of breast cancer patients often produce antibodies that react with MUC1 peptides [195]. Moreover, immunization of mice with synthetic MUC1 peptides or with cells transfected with human MUC1 reduce the incidence, and delay the growth, of tumors produced by a mouse mammary epithelial tumor cell line transfected with MUC1 in a syngeneic mouse model [193, 196].

Conclusion

In epithelial cancers, many of the phenotypic markers for malignancy have been found to be mucin glycoproteins. Alterations in both the carbohydrate and protein moieties of mucin glycoproteins in colorectal cancer cells have been amply documented. However, limited information is available on the biochemical and molecular mechanisms involved in these changes. Specifically, the mechanism of altered regulation of the mucin gene family and of the multiglycosyltransferase system in malignant transformation should be elucidated. In addition, further studies are needed to elucidate the structure-function relationship of the carbohydrate and peptide moieties of mucin glycoproteins of epithelial cancer cells. Currently, mucin associated carbohydrates and peptides in epithelial cancer cells are being exploited for immunohistochemical diagnosis and prognostic assessment, serological detection, radio-immunolocalization and immunotherapy. Development of methods to modulate mucin gene regulation and carbohydrate and peptide moieties of mucin glycoprotein antigens may play an important role in the prevention and treatment of epithelial cancer.

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