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Expression patterns of type II pneumocyte apical surface glycoconjugates in lung adenocarcinoma cells

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Abstract Monoclonal antibodies and lectins were used to examine the expression patterns of apical membrane oligosaccharide sequences specific to type II pneumocytes in atypical adenomatous hyperplasia (AAH) and lung cancer. Atypical cells of AAH and papillary adenocarcinoma cells expressed abundant sialyl Thomsen-Friedenreich (TF) antigen: this was not observed in acinar adenocarcinoma, bronchioloalveolar carcinoma with mucin production or squamous cell carcinoma. Sialyl Tn antigens was also detected on a few cells in AAH and papillary adenocarcinomas. Asialo TF and Tn antigen were not observed on the surface of carcinoma cells of any type. Alpha(α)2,3-linked sialic acids predominated in type II pneumocyte, AAH and papillary adenocarcinoma, whereas ciliated columnar cells expressed α2,6-linked sialic acids. Lewis^x and sialyl Lewis^x antigens capped the TF antigen in both *O*- and *N*-linked side chains on the surface of AAH and papillary adenocarcinoma cells, but were not expressed by type II pneumocytes. The findings demonstrate that papillary adenocarcinoma cells resemble type II pneumocytes in that they express abundant sialyl TF surface antigen, but they also express TF-related antigens not found in type II pneumocytes. Apical surface glycoconjugates of AAH have structural characteristics shared by both type II pneumocytes and papillary adenocarcinoma cells.

Key words Lung adenocarcinoma · Type II neumocyte · Thomsen-Friedenreich antigen · *Maclura pomifera* agglutinin · Sialyl Lewis

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Introduction

Both surfactant apoprotein and the sialyl Thomsen-Friedenreich (TF) antigen can be used as specific markers for type II pneumocytes in specimens of normal and pathologic lung [3, 8]. The sialyl TF antigen has been detected on the apical surface of type II pneumocytes in several mammalian species, and this allows comparative studies among different species [8].

The TF antigen is expressed frequently in various carcinomas, especially lung and breast cancers [25], and is considered to be a carcinoma-associated glycoconjugate [6]. The expression of asialo TF antigen as a nonreducing terminal saccharide sequence on carcinoma cells has been shown to elicit carcinocidal responses mediated by binding of autoantibodies against TF antigen in the presence of complement [25, 27].

The relationship between TF-related antigens and lung carcinoma is thus a significant one and we here report the expression patterns of asialo and sialyl TF antigen and related antigens in normal lung tissues, precancerous lesions and carcinomas. Expression was assessed by binding studies with various monoclonal antibodies and lectins.

Materials and methods

Thirty-five cases of primary lung cancer [21 of adenocarcinoma, 14 of squamous cell carcinoma (SCC)], 5 cases of atypical adenomatous hyperplasia (AAH) and 4 cases of squamous metaplasia were examined in this study. Tissues were fixed immediately by injecting 10% buffered formalin into the bronchial trees following by immersion in the same fixative for 24 h. The cut ends of the bronchi and samples of normal lung tissue far distant from the primary lesions were also collected. Each specimen was dehydrated through graded alcohols, cleared in xylene and embedded in paraffin. Serial sections 3 μm thick were stained with haematoxylin & eosin (HE) for survey by routine morphology, and selected specimens were stained using the methods outlined below.

Staining with colloidal iron (CI) stain at pH 2.5 was performed to identify sialic acids, which are especially abundant on the apical surface of type II pneumocytes. The high-iron diamine stain (HID) was used to detect sulfomucins. The combined Alcian blue and periodic acid–Schiff stain (AB-PAS) and Ota's dual staining method employing galactose oxidase–cold thionin Schiff (GOCTS) fol-

lowed by the paradoxical concanavalin A (PCS) method were performed to classify mucins [11, 18]. Among the lectins used in this study, those from *Maclura pomifera* (MPA) and peanut (PNA) were chosen because of their affinity for saccharides in the apical membrane of type II pneumocytes. The lectins from *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA) were employed to recognize α2–3 and α2–6 linked sialic acid residues, respectively. The lectins, conjugated to horseradish peroxidase (HRP), were purchased from EY Laboratories (San Mateo, Calif.). Immunostaining was performed with monoclonal antibodies (MAbs) against TF, Tn and sialyl Tn (STn) antigens (DAKO, Grostrup, Denmark), Lewis^x (Le^x) and sialyl Lewis^x (SLe^x) (Becton Dickinson, Ont.) and surfactant apoprotein A (SA; a gift from Dr. Toyoaki Akino, Department of Biochemistry, Sapporo Medical University, Sapporo, Japan).

For lectin histochemistry and immunohistochemistry, tissue sections were deparaffinized and then incubated in 0.3% hydrogen peroxide in methanol for 30 min to remove endogenous peroxidase activity. Sections were then incubated in phosphate-buffered saline (PBS) containing either an HRP-conjugated lectin (MPA, 5 μg/ml; PNA, 30 μg/ml; SNA, 5 μg/ml and MAA, 5 μg/ml) or a MAb (anti-TF, 1:1000; anti-Tn, 1:100; anti-STn, 1:100; anti-Le^x, 1:100; anti-SLe^x, 1:100 and anti-SA, 1:100) at 4° C for 18 h. When called for, sections were pretreated with sialidase from *Arthrobactor ureafaciens* (Nakarai Chemicals, Kyoto, Japan) (1 U/ml in 0.05 M phosphate buffer, pH 7.0) for 4 h at 37° C prior to exposure to lectins or MAbs. After rinsing in PBS, the sections incubated with MAbs were floated with HRP-conjugated goat anti-mouse IgG (Dako) diluted 1:100 in PBS for 1 h at room temperature. After further rinsing in PBS, antibody- or lectin-binding sites were visualized by incubation in peroxidase substrate solution (0.05 M phosphate buffer, pH 7.4, containing 0.01% 3,3′diaminobenzidine (Wako Pure Chemical, Tokyo, Japan) and 0.005% hydrogen peroxide) for 10 min. The sections were then counterstained with haematoxylin.

Control procedures for lectin staining consisted in incubation of the sections in labelled lectins at the concentrations indicated in solutions containing 0.1 M *N*-acetylgalactosamine (GalNAc) for MPA, 0.1 M galactose (Gal) for PNA, 0.1 mM 6′sialyllactose for SNA or 5 mM 3′sialyllactose for MAA, 0.1 M glucose, 0.1 M fucose (Fuc), 0.1 M mannose or 0.1 M *N*-acetylglucosamine (GlcNAc). Control procedures for MAb immunostaining included eliminating each stage of the staining sequence independently of the others and replacing the primary antibody with normal mouse serum.

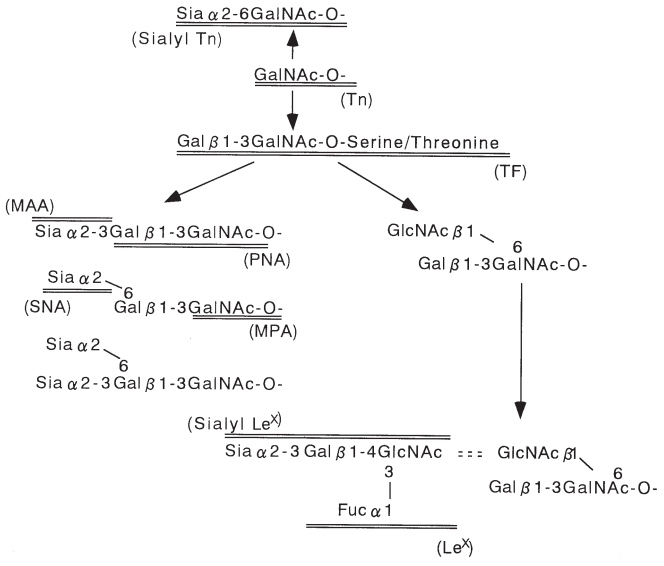


Fig. 1 Determinants of monoclonal antibodies and lectins. (Based on [22]). *Sia*, sialic acid; *GalNAc*, N-acetylgalactosamine; *Gal*, galactose; *GlcNAc*, N-acetylglucosamine; *Le^x*, *Lewis^X*; *TF*, Thomsen Friedenreich; *PNA*, peanut agglutinin; *MPA*, *Maclura pomifera* agglutinin; *MAA*, *Maackia amurensis* agglutinin; *SNA*, *Sambucus nigra* agglutinin

Results

The binding specificities of the MAbs and lectins used in this study are summarized in Fig. 1.

The results of histochemical staining reactions against four types of epithelial cells: types I and II pneumocytes, nonciliated bronchiolar cells (Clara cells) and ciliated columnar cells (ciliated cells), in normal human lung specimens are summarized in Table 1.

Colloidal iron at pH 2.5 stained the apical surface of type II pneumocytes, Clara cells and some ciliated cells. The blue coloration imparted by Cl was abolished by sialidase digestion except for that in the ciliated cells. The GOCTS-PCS sequence failed to stain any cells in regions of normal lung.

PNA stained the apical surface of type II pneumocytes and Clara cells only after sialidase digestion. MPA reacted with the surface of type II pneumocytes and Clara cells; sialidase digestion enhanced this staining (Fig. 2). SNA reacted with more than 50% of ciliated cells and with a few type II pneumocytes and Clara cells. Digestion with sialidase, paradoxically, enhanced SNA binding. MAA stained all types of epithelial cells, and this reactivity was abolished by sialidase digestion.

Sialidase digestion revealed cryptic TF antigen expressed at the apical surface of all type II pneumocytes (Fig. 3) and a few Clara cells. The antibody against Tn reacted strongly with granules in the cytoplasm of some type II pneumocytes and ciliated cells. Anti-STn, however, stained the apical surface of a few ciliated cells, but not that of type II pneumocytes.

Anti-Le^x and anti-SLe^x showed no affinity for any cells in peripheral regions of the lung. Anti-SA was observed in the cytoplasm of most type II pneumocytes and of a few Clara cells.

Atypical cells of AAH showed reactivity very similar to that of type II pneumocytes in the normal lung tissue (Fig. 4). Tn, STn, Le^x and SLe^x were detected in less than 1–10% of atypical cells.

The adenocarcinomas examined included 2 cases of bronchioalveolar carcinoma (BAC) without mucin production, 5 BAC with mucin production, and 2 acinar adenocarcinomas [30]. BACs without mucin production were contained in the papillary adenocarcinomas, because it is often difficult to distinguish the former from the latter morphologically. BACs with mucin production (mucinous BACs) and acinar adenocarcinomas failed to stain for TF and its associated antigens. The AB-PAS procedure revealed abundant mucin in all cases of mucinous BAC, and the GOCTS-PCS sequence showed that 3 of 5 mucinous BACs possessed both GOCTS-positive and PCS-positive mucin.

Table 1 Histochemical reactivities of normal epithelial cells in the bronchiolar and alveolar regions. The number of positive cells (distribution on score): 0; no cells, 1; <1%, 2; 1–10%, 3; 10–50%, 4; 50–90%, 5; >90% (*CI* colloidal iron, *Si-* sialidase digestion, *Type I cell* type I pneumocyte, *Type II cell* type II pneumocyte, *Clara cell* non-ciliated bronchiolar cell, *Ciliated cell* ciliated columnar cell, *AAH* atypical adenomatous hyperplasia, *Pap. Adeno*. papillary adenocarcinoma, *SCC* squamous cell carcinoma, *S* surface was predominantly stained, *C* cytoplasm was predominantly stained, *W* very weakly stained)

	CI	Si-CI	MPA	Si-MPA	PNA	Si-PNA	SNA	Si-SNA	MAA	Si-MAA	TF	Si-TF	Tn	STn	Le^x	SLe^x	SA
Type II cell	5S	O	4S	5S	1S	5S	1S	5S	5S	O	O	5S	2C	O	O	O	5C
Type I cell	W	O	O	O	1S	O	O	5S	5S	O	O	O	O	O	O	O	O
Clara cell	5S	O	1S	4S	1S	5S	1S	5S	3S	O	O	1S	O	O	O	O	2C
Ciliated cell	5S	3S	W	W	W	4S	4S	5S	4S	W	O	2S	2C	2S	O	O	O
AAH	5S	O	5S	5S	4S	5S	2S	5S	4S	O	O	5S	4C	1S	1S	1S	5C
Pap. Adeno.	3–5S	O	2–4S	3–5S	1–4S	3–5S	1–3S	3–5S	3–5S	1–3S	O	2–5S	1–4C	1–2S	2–5S	2–5S	1–4C
SCC	O	O	O	W	O	W	O	W	W	O	O	O	O	O	O	O	O

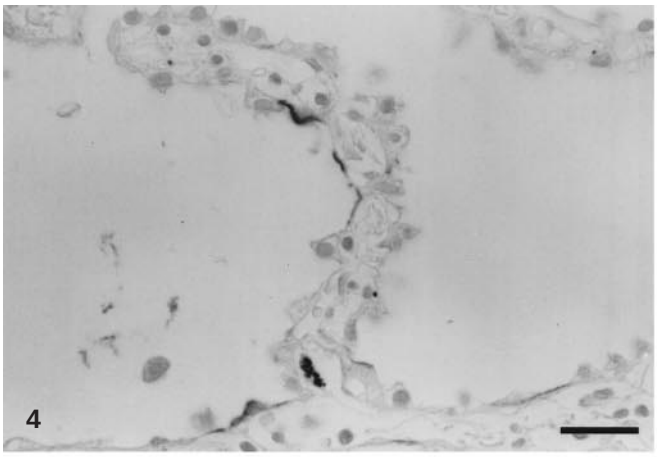
Fig. 2 Normal alveoli. The apical surface of all type II pneumocytes was stained. MPA, ×400, *bar* 25 μm

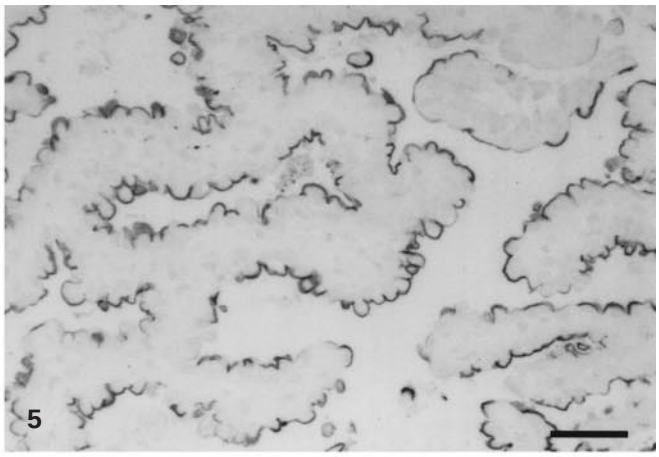
Fig. 3 Normal alveoli. The apical surface of all type II pneumocytes was stained. Sialidase digestion-TF stain; ×400, *bar* 25 μm

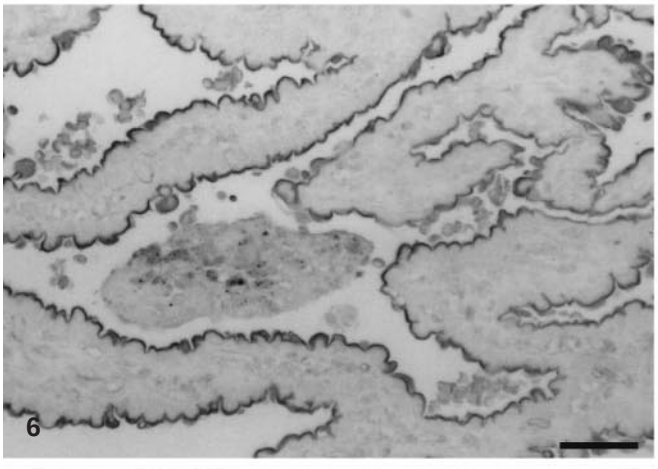
The 14 cases of papillary adenocarcinoma were divided into three groups: 7 well-differentiated, 6 moderately and 1 poorly differentiated adenocarcinoma [17]. The more highly differentiated carcinoma cells tended to stain similarly to normal type II pneumocytes. The staining results for the papillary adenocarcinomas are summarized in Table 1 and described in more detail.

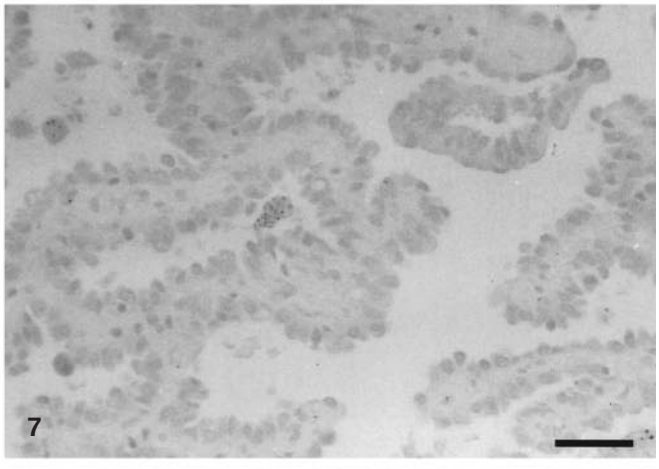
Colloidal iron at pH 2.5 stained the apical surface and cytoplasmic granules of at least 10% of carcinoma cells in all cases. Sialidase digestion mostly abolished the surface staining in most cells, although some reaction remained with Cl stained. The persisting Cl stain corresponded to positive HID reactivity. AB-PAS staining showed only a small amount of stored mucin, and PCS-positive mucin was not observed.

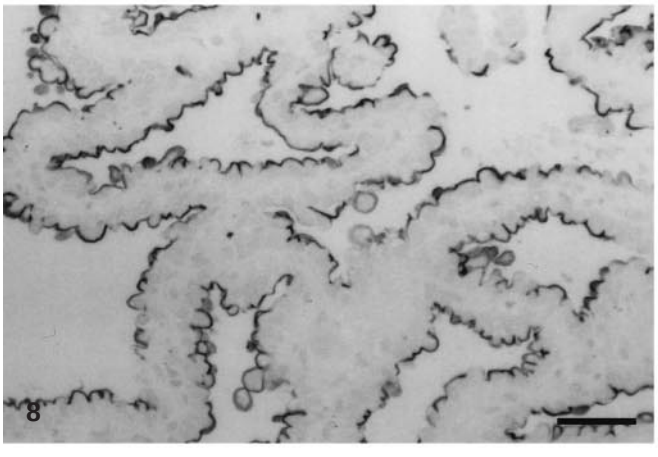
In the absence of sialidase digestion PNA stained under 1% of cells, whereas MPA reacted with 50–90% of the tumour cells. Both PNA- and MPA-positive carcinoma cells increased in number after sialidase digestion.

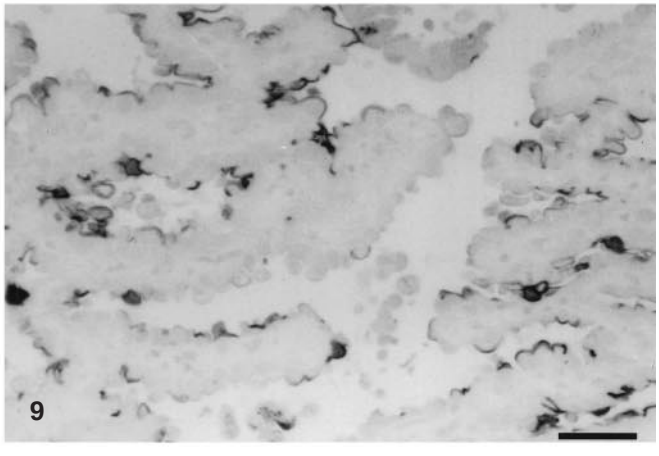












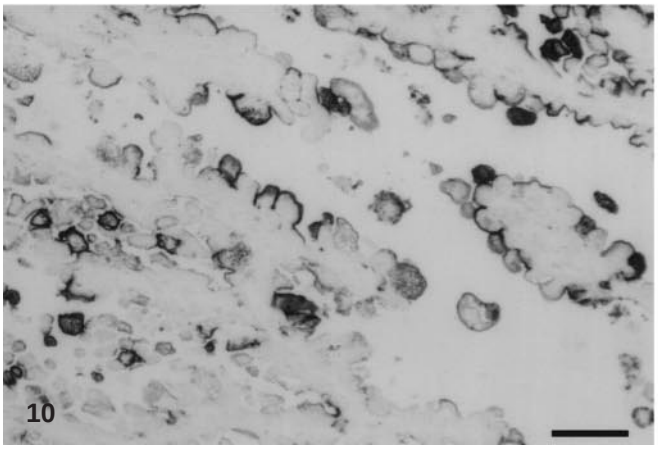


Fig. 4 Atypical adenomatous hyperplasia. The apical surface of a few atypical cells was stained. Anti-STn stain, ×400, *bar* 25 μm

Fig. 5 Adenocarcinoma. The apical surface of adenocarcinoma cells was stained. Sialidase digestion-MPA, ×200, *bar* 50 μm

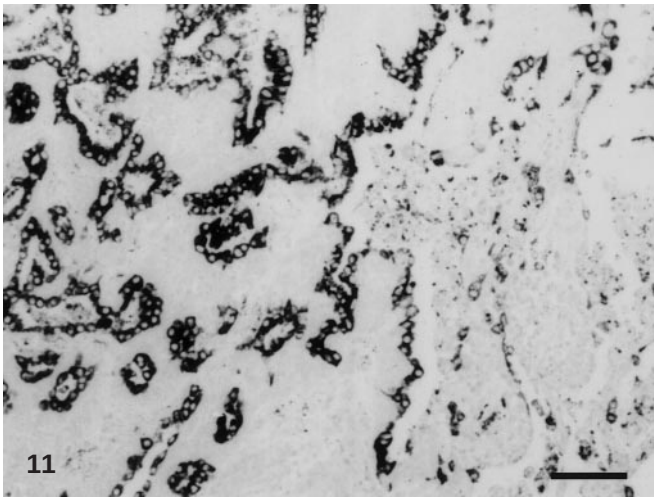
Fig. 6 Adenocarcinoma. The apical surface of adenocarcinoma cells was stained. MAA, ×200, *bar* 50 μm

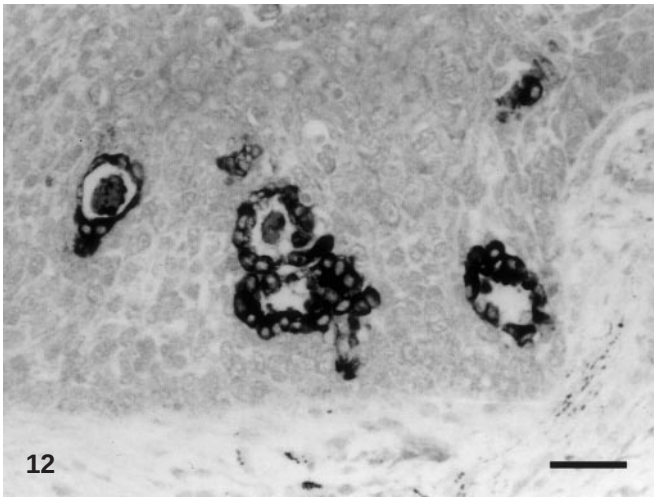
Fig. 7 Adenocarcinoma. No carcinoma cells were stained. Anti-TF stain, ×200, *bar* 50 μm

Fig. 8 Adenocarcinoma. The apical surface of adenocarcinoma cells was stained. Sialidase digestion-TF stain, ×200, *bar* 50 μm

Fig. 9 Adenocarcinoma. The apical surface of some adenocarcinoma cells was stained. Anti-STn stain, ×200, *bar* 50 μm

Fig. 10 Adenocarcinoma. The apical surface of some adenocarcinoma cells was stained. Anti-SLe^x stain, ×200, *bar* 50 μm





Figs. 11, 12 Squamous cell carcinoma. The cells forming the acinar structure in the carcinoma tissues were stained. Anti-surfactant apoprotein A stain, **Fig. 11** ×100, *bar* 100 μm and **Fig. 12** ×200, *bar* 50 μm

PNA and MPA showed reactivity for more carcinoma cells than were stained by anti-TF antibody after sialidase digestion (Fig. 5). SNA stained the apical surface of the carcinoma cells in all cases, although staining was weak in more than half of them. More carcinoma cells were stained strongly after sialidase digestion. MAA stained 10–90% of carcinoma cells in all cases (Fig. 6). MAA binding decreased after sialidase digestion, although some cells did not lose reactivity.

The antibody against TF showed no affinity for carcinoma cells (Fig. 7), whereas sialidase digestion exposed epitopes reactive for this antibody on the apical surface of from a few to more than 90% of carcinoma cells in all cases (Fig. 8). Anti-Tn antibody demonstrated affinity for granules in the cytoplasm of some (from a few to 90%) carcinoma cells in all cases. Most of the sialyl TF-positive cells were also anti-Tn positive, although sialyl TF-negative and anti-Tn-positive carcinoma cells were observed and vice versa. Anti-STn stained the apical surface of some carcinoma cells in all except 2 cases (Fig. 9). Most anti-STn-positive carcinoma cells were also sialyl TF positive, although some STn-positive cells were sialyl TF negative.

Anti-Le^x stained the apical surface or cytoplasm of some (from a few to more than 90%) carcinoma cells. Anti-SLe^x (Fig. 10) was reactive for a greater number of carcinoma cells reacted with anti-Le^x. However, there was no correlation between Le^x- and/or SLe^x-positive carcinoma cells and sialyl TF-positive ones.

Anti-SA stained the cytoplasm of a few up to more than 90% of carcinoma cells in all cases. There was no correlation between SA-positive and sialyl TF-positive carcinoma cells.

Keratinizing SCC cells often showed nonspecific reactivity, so that the results reported here pertain only to nonkeratinized SCC cells. Cells that formed acinar structures and showed the same histochemical reactivities as the type II pneumocytes in healthy lung tissue (Figs. 11, 12) were interpreted as normal type II pneumocytes remaining in tumours.

As shown in Table 1, areas of squamous metaplasia and squamous cell carcinoma cells revealed no significant reactivity for the probes employed in this study.

No staining was present in sections incubated in the unlabelled lectins or a solution of HRP in place of lectin-HRP conjugates. Staining with MPA-HRP was inhibited almost totally by addition of 0.1 M Gal or 0.1 M GalNAc to the lectin solution. Binding of PNA-HRP was abolished by preincubation with 0.1 M Gal and partially inhibited by 0.1 M GalNAc. Reacting with SNA-HRP was almost completely inhibited by addition of 0.1 mM 6′sialyllactose, whereas 5 mM 3′sialyllactose had no effect on staining with lectin. Staining with MAA-HRP was abolished by preincubation with 0.1 mM 3′sialyllactose, although 5 mM 6′sialyllactose had no effect. No staining was seen in any control section for immunostaining.

Discussion

Sialyl TF antigen, which appears to serve as an excellent marker of normal type II pneumocytes regardless of species [3, 8], was present on the apical surface of papillary adenocarcinoma cells. These cells also showed cytoplasmic and surface staining for Tn and sialyl Tn antigens, neither of which was observed in type II pneumocytes of healthy lung tissue. Other types of adenocarcinoma, including acinar adenocarcinoma and BAC with mucin production and SCC, failed to stain with probes for the sialyl TF and TF-related antigens.

The lectin probes used in this study revealed that normal type II pneumocytes and Clara cells express the same terminal trisaccharide of NeuAc α2–3 Gal β1–3GalNAc at the apical surface. Following sialidase digestion the antibody against TF antigen recognized all type II pneumocytes, but reacted with a few Clara cells. A minor difference in carbohydrate structure recognized by anti-TF antibody or steric hindrance by other sugar

structures might explain this discrepancy. Ciliated columnar cells had sialomucin containing both α2,3- and α2,6-linked sialic acids, and sulfomucin on their apical surface. Sialyl Tn antigen, which was observed in AAH and papillary adenocarcinoma, was detected in some ciliated columnar cells in addition to Tn antigen and sialyl TF antigen. This may be an aberrant expression of the sialyl Tn antigen, because no carcinoma cells showed morphological differentiation toward the ciliated cells.

The histochemical properties of carcinoma cells may reflect those of their progenitor cells. Variable positivity, up to 90% of papillary adenocarcinoma cells also being reactive for antibody against SA antigen in all cases, was found, so that most papillary adenocarcinomas were classifiable as type II pneumocyte- and/or Clara cell-type tumours within the ultrastructural classification of lung cancer [12, 23]. BAC with mucin production, which might correspond to the goblet cell type, showed different reactivities from the papillary adenocarcinomas with scanty mucin. Three cases of BAC showed organoid differentiation, simulating gastric pyloric mucosa by mucin histochemistry [9]. PCS-positive mucin specific for the gastric-type BAC was never detected in normal lung tissues or other types of lung cancer. Acinar adenocarcinomas, which are relatively rare and mostly occur in the large bronchi [30], did not contain the TF and sialyl TF antigens. These lesions showed predominantly glandular structures, suggesting a relationship with the bronchial glands.

Le^x and sialyl Le^x were present only in papillary adenocarcinomas. TF is an important backbone structure of Le^x and sialyl Le^x at the terminal portion of the *O*-glycan. TF and its related antigens are *O*-glycans and include core 1 backbone structures. Le^x and sialyl Le^x structures are also added to *O*-glycan with core 2 backbone structure in addition to *N*-glycan [4]. Core 2 structures were synthesized by attaching GlcNAc to the GalNAc of TF via β1–6 linkage. The β1–6 GlcNAc transferase, which is a key enzyme for the synthesis of core 2 structures, has been shown to be significantly elevated in myelogenous leukaemia relative to normal granulocytes [2, 4]. Moreover, mRNA encoding this enzyme was expressed in colonic carcinoma cells, but not in normal colonic epithelium [22]. Thus, it is possible that this β1–6 GlcNAc transferase also plays an important part in the pathogenesis of papillary adenocarcinoma of the lung.

Sialyl TF-positive and SA-positive carcinoma cells were observed in papillary adenocarcinoma, and both sialyl TF-positive, SA-negative cells and TF-negative, SA-positive cells were also observed. Expression of sialyl TF antigen and its related antigens was independent of SA expression in papillary adenocarcinomas. Sialyl TF antigen may thus be another useful marker for this type of lung adenocarcinoma, for which SA has thus far been the only marker available. Sialyl TF antigen has also been detected on the apical surface of type II pneumocytes in mammalian species, including rabbit and sheep [8]. A condition similar to BAC occurs in several species, and ovine pulmonary adenomatosis is well known [19]. Sialyl TF antigen may be retained on the apical surface of adenoma or carcinoma cells in ovine pulmonary adenomatosis.

Sialic acids capping TF and related antigens on the surface of the normal type II pneumocytes and papillary carcinoma cells may play an important part in protection against their recognition by endogenous antibodies against TF and Tn antigens [25]. A thick mucous layer containing abundant sialic acids coats the apical surface of type II pneumocytes [7, 10]. In both the human [5] and rat [7] lung, terminal sialic acids are added to PNA-reactive sugar structures on the apical surface of type II pneumocytes during postnatal development. In human tissues, TF antigen is expressed only in the parenchyma of the brain and on spermatocytes, which are an immunoprivilege site [3], because naturally occurring antibodies against TF antigen are cytotoxic in the presence of complement [26]. TF and Tn antigen vaccination therapy has been effective in preventing recurrence of advanced breast cancer [28].

Sialic acids linked α2,3 were abundantly present on the surface of type II pneumocytes and papillary adenocarcinoma cells, whereas those linked α2,6 were present on ciliated cells. Sialylated glycoconjugates function as cell-surface receptors for bacteria, viruses, and mycoplasma [20, 24] and as antigenic immunodeterminants, in addition to providing protection from the host immune reaction mentioned above. It is interesting that the cell coat expression of sialic acids in the bronchial region is different from that in the alveolar region. α2,6-linked sialic acids possibly show more affinity for pathogens causing bronchial infection than for those causing pneumonia.

SNA has been shown to bind with high affinity to glycoconjugates containing the terminal sequence Neu-Ac α2–6 Gal/GalNAc [21], although the SNA reactivity with type II pneumocytes and papillary adenocarcinoma cells was intensified paradoxically after digestion with sialidase. SNA also shows affinity for terminal GalNAc and asialofetuin (Gal β1–3GalNAc α-R) [29], and SNA may thus bind TF antigen exposed by sialidase digestion.

Affinity for Cl in type II pneumocytes was abolished by sialidase digestion, although it was only partially eliminated in carcinoma cells. HID showed the same reactivity as the Cl stain after sialidase digestion, indicating that glycoconjugates in the carcinoma cells are sulfated. MAA-positive but Cl-negative carcinoma cells were observed after sialidase digestion. MAA is a lectin specific to α2,3-linked sialic acid [14]. It is possible that sensitivity of MAA for sialic acids is higher than that of Cl, or that MAA reacts with epitopes other than sialic acids.

Results obtained with MPA and PNA mostly support anti-TF staining. MPA or PNA-reactivity has often been used a marker for type II pneumocytes [1, 5, 15, 16], and both lectins also recognize Clara cells of several mammalian species including humans [8]. MPA and PNA were thought to react with terminal GalNAc and Gal-GalNAc, respectively. They did not, however, show the

same binding pattern as the anti-TF antibody. They stained a greater number of carcinoma cells than those reactive with anti-TF, possibly owing to reactivity with epitopes other than the TF antigen.

Atypical cells of AAH, which is a putative precursor of adenocarcinoma [13], largely showed the same reactivity as normal type II pneumocytes, although a few of the atypical cells also demonstrated sialyl Tn, Le^x or sialyl Le^x epitopes. Atypical cells in AAH had staining characters intermediate between the normal type II pneumocyte and papillary adenocarcinomas, perhaps indicating that AAH represents a precancerous precursor of papillary adenocarcinoma. Well-differentiated papillary adenocarcinomas were more likely to be positive for sialyl TF antigen than were moderately and poorly differentiated tumours. Thus, differentiation may be related to the expression of the sialyl TF antigen.

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