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Mucin-associated T antigens in benign and malignant epithelium of the gall bladder, extrahepatic bile ducts and ampulla of Vater

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The mucin-associated antigens Tn, sialosyl-Tn (STn), T and sialosyl-T (STAg) antigens accumulate through aberrant and incomplete glycosylation in malignant epithelial cells. Their diagnostic and prognostic significance in tumours of the colon and cervix has been described, and a possible role for Tn antigen in cell-to-cell adhesion has been suggested. These antigens have been demonstrated through peanut agglutinin (PNA) lectin binding and more recently using specific monoclonal antisera. Differences between the two methods have been described, which may be due to fixation schedules and/or specificity. We have investigated the effect of fixation on the binding of biotinylated PNA lectin and compared its reactivity with the immunoreactivity of monoclonal antisera to Tn, STn, T and STAg antigens in benign and malignant epithelium of the gall bladder, extrahepatic bile ducts and ampulla of Vater. We found that short-term fixation in formol sublimate resulted in poor PNA binding. All other tested fixation schedules showed strong perinuclear binding, similar to that found on cryostat sections. When compared with monoclonal antisera, PNA binding demonstrated the lowest specificity in benign epithelium. In both benign and malignant epithelium, the two methods cannot substitute for each other. STn and STAg antigens were found to be oncodevelopmental throughout the extrahepatic biliary tract. When used in a panel, they are useful as diagnostic markers of malignancy in gall bladder epithelium.

Keywords: extrahepatic biliary tract carcinoma, gall bladder carcinoma, T antigen

Introduction

Mucin-associated antigens are formed following the stepwise addition of *N*-acetylgalactosamine (GalNAc), sialic acid (NeuAc), galactose (Gal) and further NeuAc molecules to serine or threonine residues on the polypeptide backbone of glycoproteins. These form the Tn, sialosyl-Tn (STn), T (TAg) and sialosyl-T (STAg) antigens respectively (Table 1). Additions to the backbone structure are controlled through the expression of specific glycosyltransferases [4]. Accumulation of these core oligosaccharides through aberrant and incomplete glycosylation is a feature of malignant epithelial cells [5–7].

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The diagnostic and prognostic significance of mucin-associated antigens has been highlighted in recent literature. STn antigen immunoreactivity has been suggested as a diagnostic and prognostic indicator in colorectal carcinoma [1, 8], and mucin carbohydrate content correlates with the biological behaviour of pancreatic tumours [9]. Tn antigen expression in cervical cancer is also reported to be a useful prognostic indicator [10].

Peanut agglutinin (PNA) from *Arachis hypogaea* detects T antigen through its affinity for the disaccharide sequence D-galactose-β-(1,3)-*N*-acetylgalactosamine [3]. This disaccharide, then, lacks the addition of NeuAc molecules, and its binding to PNA is indicative of incomplete glycoprotein synthesis in colonic epithelium [11]. However, there have been reports that results using lectin

Table 1. Carbohydrate epitope structures of the antisera used in this study [1, 2]

Antigen	Structure
Tn	GalNAc–0–Ser/Thr
Sialosyl-Tn	NeurAcα2–6 GalNAc–0–Ser/Thr
T	Galβ1–3 GalNAc–0–Ser/Thr

Note: PNA binds preferentially to the disaccharide of the T antigen but also binds to the monosaccharide galactose [3].

histochemistry do not agree with those obtained when specific antisera to T antigen are used [5, 12].

We have investigated the binding of a biotinylated PNA and the immunoreactivity of antisera to Tn, STn and TAg antigens in benign and malignant epithelium of the gall bladder, extrahepatic bile ducts and ampulla of Vater. Sialosyl-TAg and cryptic TAg (PNA binding) were investigated following neuraminidase pretreatment.

As fixation can have an effect on lectin histochemistry [13] we have also investigated various fixation schedules and their effect on the binding of PNA to inflamed gall bladder epithelium.

Materials and methods

Twenty-three cases of inflamed and eight cases of dysplastic epithelium of the gall bladder, along with six cases of inflammation of the biliary tract (three extrahepatic bile duct, three ampulla of Vater) and 55 carcinomas (19 gall bladder, 18 extrahepatic bile duct and 18 carcinomas of the ampulla of Vater) were taken from file. The majority of these samples had been fixed in unbuffered formol saline for 12 h followed by histology fixative for 4 h (Table 2).

Serial paraffin wax-embedded sections were cut at 4 μm from all cases and heat dried at 56°C overnight. Sections were stained with haematoxylin and eosin. Lectin-binding glycoproteins were investigated using biotinylated PNA (Vector Labs) 5 μg/ml in 10 mM HEPES-buffered saline and localized using peroxidase–streptavidin–biotin complex (StABC, Dako).

Immunoreactivity to the antisera for Tn, STn and T antigens was demonstrated. All primary antisera were diluted to 1:100 (0.91 μg/ml, Dako) in 0.05 M Tris-buffered saline and localized using a standard peroxidase StABC (Dako) method.

Table 2. Fixation protocol for blocks obtained from fundus of gall bladders removed for cholecystitis

Fixative	Duration (h)
Snap frozen in liquid nitrogen	Stored at −20°C
Histology fixative	4
Formol sublimate	4
Unbuffered formol saline	12
The above block was bisected to give further blocks which were subsequently fixed for the time indicated below	
Histology fixative	4
Formol sublimate	4
Unbuffered formol saline	4

Note: Histology fixative is composed of unbuffered formol saline containing phosphotungstic, calcium chloride and lithium carbonate at 0.002% (w/v).

STAg and cryptic TAg (SPNA) were investigated following neuraminidase pretreatment (type III from *Vibrio cholerae* Sigma). The neuraminidase was diluted 1:20 in acetate buffer pH 5.5, and sections were incubated at 37°C overnight, washed in distilled water and localized through TAg immunoreactivity and PNA binding as described above.

Diaminobenzidine–hydrogen peroxide acted as the chromogen in all cases, and the sections were counterstained in haematoxylin.

The effect of fixation on PNA binding was studied using seven gall bladder specimens, removed for cholecystitis. Blocks of fundus were fixed according to the fixation schedule described in Table 2. In paraffin wax-embedded sections, PNA binding was demonstrated as described above. Cryostat sections were cut at 5 μm, fixed in acetone for 20 min, air dried and stained by localizing the biotinylated lectin using the StABC method as described above.

Two observers independently scored the results. A positive reaction was recorded when 20% or more of the epithelium/tumour showed lectin binding or immunoreactivity.

Inhibition of PNA binding using 0.2 M D-galactose (Aldrich) acted as a negative control. Selected positive cases in which the primary antiserum was replaced by mouse IgG1 (STn) and mouse IgM (Tn and TAg, 0.91 μg/ml, Dako) acted as negative immunohistochemical controls. The presence on red blood cells of T antigen following neuraminidase pretreatment (sialosyl-T antigen) acted as an internal positive control.

Results

Haematoxylin and eosin-stained sections confirmed that all tumours were adenocarcinomas. These were graded as in Tables 3–5. The age of the patients presenting with tumours of the gall bladder ranged from 45 to 86 years with a mean of 70 years and a male–female ratio of 1:5.3. The ages of patients with extrahepatic bile duct adenocarcinomas ranged from 54 to 84 years with a mean of 66 years and a male–female ratio of 1:2.6. The ages of patients with tumours of the ampulla of Vater ranged from 45 to 82 years with a mean of 66 years and a male–female ratio of 1:1.6 years.

In the fixation study, all blocks showed supranuclear PNA binding with focal binding along the apical membrane. The apparent intensity varied from case to case, but was consistent within each case. Only blocks fixed in formol sublimate for 4 h showed poor staining in all cases.

Supranuclear binding of PNA was present in inflamed and dysplastic epithelium of the gall bladder, extrahepatic bile ducts and ampulla of Vater (Tables 3–5).

PNA binding was present in adenocarcinomas of the gall bladder, extrahepatic bile ducts and ampulla of Vater (Tables 3–5). In benign epithelium and well-differentiated tumours, this was seen in a

Table 3. Expression of Tn, sialosyl-Tn, T antigen and sialosyl T antigen in benign and malignant epithelium of the gall bladder

Grade	*n*	Tn	STn	TAg	S-TAg	PNA	SPNA
Inflamed	23	11 (47.8)	0	0	0	17 (73.9)	22 (95.7)
Dysplastic	8	7 (87.5)	0	0	0	8 (100)	8 (100)
Carcinoma	19	15 (78.9)	13 (68.4)	3 (15.8)	13 (68.4)	17 (89.5)	18 (94.7)
Papillary	2	2 (100)	2 (100)	1 (50)	2 (100)	2 (100)	2 (100)
Well-differentiated	4	3 (75)	3 (75)	1 (25)	3 (75)	4 (100)	4 (100)
Moderately differentiated	8	5 (62.5)	5 (62.5)	0	4 (50)	7 (87.5)	7 (89.5)
Poorly differentiated	5	5 (100)	3 (60)	1 (20)	4 (80)	4 (80)	5 (100)

Note: Figures in brackets denote the percentage of positive cases (> 20% positive epithelium). STAg and SPNA denote TAg immunoreactivity and PNA binding following neuraminidase pretreatment.

Table 4. Expression of Tn, sialosyl-Tn, T antigen and sialosyl-T antigen in benign and malignant epithelium of the extrahepatic bile ducts

Grade	*n*	Tn	STn	TAg	STAg	PNA	SPNA
Inflamed	3	3 (100)	0	0	0	3 (100)	3 (100)
Carcinoma	18	9 (50)	3 (16.7)	0	8 (44.4)	15 (83.3)	15 (83.3)
Well-differentiated	8	4 (50)	3 (37.5)	0	3 (37.5)	7 (87.5)	7 (87.5)
Moderately differentiated	8	4 (50)	0	0	3 (37.5)	6 (75)	6 (75)
Poorly differentiated	2	1 (50)	0	0	0	2 (100)	2 (100)

Note: Figures in brackets denote the percentage of positive cases (> 20% positive epithelium). STAg and SPNA denote TAg immunoreactivity and PNA binding following neuraminidase pretreatment.

Table 5. Expression of Tn, sialosyl-Tn, T antigen and sialosyl-T antigen in benign and malignant epithelium of the ampulla of Vater

Grade	*n*	Tn	STn	TAg	STAg	PNA	SPNA
Inflamed	3	2 (66.7)	0	0	0	3 (100)	3 (100)
Carcinoma	18	11 (61)	6 (33.3)	0	7 (38.9)	15 (83.3)	16 (88.9)
Well differentiated	4	3 (75)	1 (25)	0	1 (25)	3 (75)	4 (100)
Moderately differentiated	10	5 (50)	3 (30)	0	3 (30)	9 (90)	9 (90)
Poorly differentiated	4	3 (75)	2 (50)	0	3 (75)	3 (75)	3 (75)

Note: Figures in brackets denote the percentage of positive cases (> 20% positive epithelium). STAg and SPNA denote TAg immunoreactivity and PNA binding following neuraminidase pretreatment.

supranuclear pattern (Figure 1). However, poorly differentiated tumour cells showed a granular cytoplasmic binding pattern. In some cases, poorly differentiated elements of moderately differentiated adenocarcinoma failed to bind PNA, even after neuraminidase pretreatment.

When present, all antigens were seen to be associated with the supranuclear area of both benign and maligant epithelium of the gall bladder and extrahepatic biliary tract. STAg expression was also seen associated with the luminal surface of malignant epithelium. Cytoplasmic immunoreactivity was seen only in poorly differentiated malignant cells (Figures 2 & 3).

Throughout the test material, PNA binding and T-antigen immunoreactivity occurred together in 19.6% of cases, PNA and Tn antigen in 58.7% of cases and PNA and STn antigen in 33.7% of cases. PNA binding was found in 61.8% of cases which also expressed Tn, STn or TAg antigens according to the distributions described above. Tn and STn antigens were coexpressed by 57.9% of gall bladder adenocarcinomas, 5.6% of extrahepatic bile duct tumours and 27.8% by tumours of the ampulla of Vater.

All controls following inhibition of PNA binding by 0.2 M D-galactose and replacing the primary antiserum by mouse IgG1 and IgM were uniformly negative.

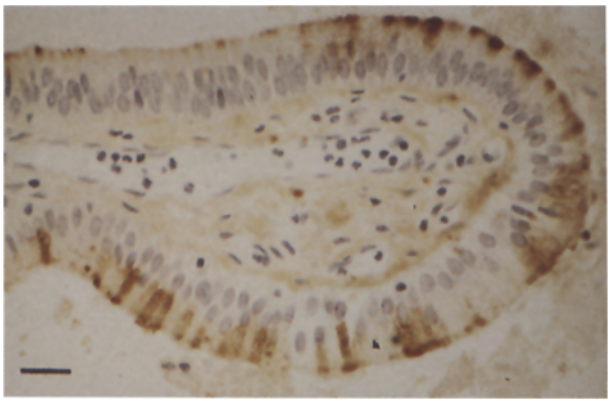

Figure 1. PNA binding in inflamed gall bladder epithelium. Bar = 25 μm.

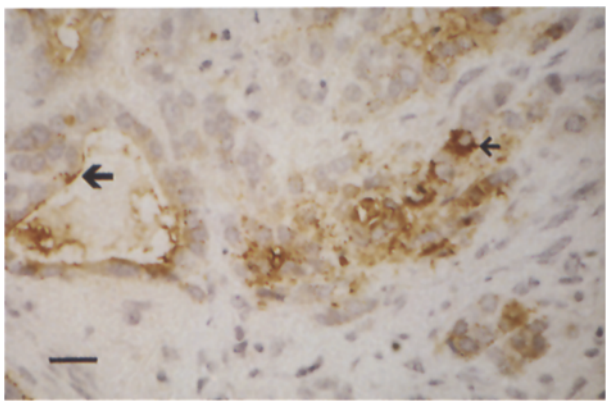

Figure 2. Sialosyl-Tn antigen in moderately differentiated adenocarcinoma of the gall bladder showing supranuclear (large arrow) and cytoplasmic immunoreactivity (small arrow). Bar = 25 μm.

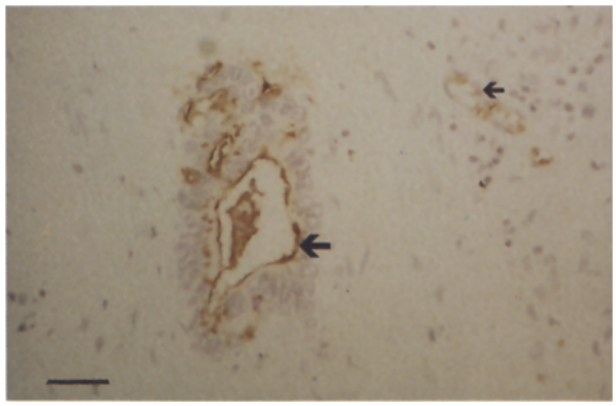

Figure 3. Sialosyl-T antigen in well-differentiated adenocarcinoma of the gall bladder showing membrane immunoreactivity (large arrow). Note the positive red blood cells (small arrow). Bar = 50 μm.

Discussion

Fixation affects lectin binding, and the different fixation schedules used by research groups may partly explain conflicting results [13]. We compared fixation schedules which can be used by such groups. Only those blocks fixed for short periods of time in formol sublimate gave inferior binding of PNA. Such a fixation schedule, however, was not used on our file material. This material therefore, was suitable for the retrospective study of PNA binding.

Supranuclear binding of PNA, in the region of the Golgi apparatus [14], has been reported in benign and malignant epithelium of the colon [15–18]. Throughout the gall bladder and extrahepatic biliary tract, the supranuclear positivity seen with PNA and mucin-associated antigens corresponds to this area of the cell. As in the colon, glycosylation of these antigens appears to be associated with the Golgi apparatus. Only poorly differentiated malignant cells showed a cytoplasmic expression of antigen.

Yuan *et al.* [12] compared the T-antigen expression in normal, premalignant and malignant human colonic tissue using lectin and antibody immunohistochemistry. PNA demonstrated the best sensitivity in cancer tissues but the lowest specificity in normal mucosa. Itzkowitz *et al.* [5] reported differences between binding of peanut lectin, a polyclonal antibody and monoclonal antibody to T antigen in colon cancer. Such differences may be due to the degree of sialylation associated with the PNA-bound structures [19]. In our study, PNA would appear to bind non-specifically when compared with T-antigen immunohistochemistry. Using kappa statistics on the results from the gall bladder and extrahepatic biliary tract, no measure of agreement was found when PNA binding was compared with TAg, Tn or STn antigen immunohistochemistry ($\kappa = 0.01$, 0.02 and 0.02 respectively). It would appear, therefore, that PNA binding and immunohistochemistry using these monoclonal antisera cannot substitute for each other.

The accumulation of the mucin-associated antigens is a feature of malignant epithelial cells [5–7]. Also, a relationship between prognosis and antigen expression in colorectal and cervical carcinomas has been described [1, 8, 10] and a possible role for Tn antigen in cell to cell adhesion has been suggested [10, 20].

We found that biotinylated PNA binds not only to malignant epithelium but also to inflamed epithelium throughout the gall bladder and extrahepatic biliary tract. In gall bladder epithelium, however, both STn and STAg antigens are oncodevelopmental. The expression of these antigens in gall bladder biopsies may therefore prove useful as a marker of malignancy.

In normal colonic epithelium, Tn and STn antigens are thought to be masked by more oligosaccharides and are revealed through incomplete glycosylation [11]. In the gall bladder and extrahepatic biliary tract, Tn antigen expression is associated with an increase in cell turnover brought about by inflammation [21]. STn antigen expression is oncodevelopmental. Itzkowitz *et al.* [5] suggested that the regulation of mucin oligosaccharides is different in normal and malignant colonocytes. Yonezawa *et al.* [6] agreed that the synthesis of STn antigen is different in normal tissues and carcinomas. They found that STn antigen expression is not restricted to adenocarcinomas but is also found in hepatocellular carcinomas, renal cell carcinomas and papillary carcinomas of the thyroid gland. Our results have also shown that neuraminidase pretreatment revealed an increase in the expression of STAg in malignant epithelium. This would suggest that the control of STn and STAg antigen expression through the up-regulation of glycosyltransferases plays a significant role in these carcinomas [4].

When used in a panel of antisera to the T-antigen family, the oncodevelopmental nature of STn and STAg antigens in gall bladder carcinomas is therefore useful as a diagnostic marker of malignancy. A relationship between prognosis and antigen expression in colorectal and cervical carcinomas has been described and a possible role in cell adhesion suggested by other researchers. Also, the up-regulation of specific glycosyltransferases may play a role in carcinomas of the gall bladder. Investigating the synthesis of the glycosyltransferases involved in mucin-associated antigen expression and their relationship to cell adhesion would be useful in elucidating the significance of our results.

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