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Cell type-dependent alterations of binding of synthetic blood group antigen-related oligosaccharides in lung cancer

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Blood group antigen-related oligosaccharides have been implicated in growth regulation, cell mobility control and adhesion; we are therefore interested in the localization of receptors for these oligosaccharides in tumour cells. Labelled neoglycoconjugates that carry synthetic sugar structures are suitable tools to determine: whether such binding sites are present in human lung cancer; whether structural alterations of the glycoligand part will affect extent of binding; and whether cell type-associated alterations can be detected. Sections from 121 cases of lung cancer, representing small cell and non-small cell lung carcinoma, mesothelioma and metastases from extrapulmonary primary carcinomas were used to study the binding of nine synthetic AH- and Le-related oligosaccharides. Probes with fucose-α1-3/4-*N*-acetylglucosamine-β1-R, an A-like disaccharide and 3′-sulfated galactose as ligand appear to bind less well to small cell than to non-small cell lung cancer cases, whereas Le^c-disaccharide distinguishes mesothelioma from metastatic carcinoma. The latter ligand, A-like disaccharide and H (type III)-like trisaccharide exhibit evident cell type-associated differences in extent of binding. Thus, tailor-made neoglycoconjugates constitute a promising class of histopathological tools that warrants further study.

Keywords: neoglycoconjugate; lectin; lung cancer; blood group antigen; tumour diagnosis

Introduction

Cellular properties are governed by recognitive interactions between various types of molecular determinants. Analysis of their expression yields information on cellular behaviour and responses. The development of histopathological tools to define tumour cell characteristics like cell lineage, status of differentiation or capacity for metastasis formation aims eventually to take advantage of relevant biochemical epitopes for diagnostic means. When a certain class of molecules has proven its physiological significance, purposeful design of appropriate markers may achieve progress in tumour pathology. A biochemical concept thus deliberately guides the preparation of markers and, subsequently, their histochemical application.

During the last decade the notion has increasingly been supported that the carbohydrate chains of cellular glycoconjugates are information-bearing molecules [1–4]. Their expression, monitored by monoclonal antibodies or other sugar receptors like plant lectins, appears to be strictly regulated [5–9]. This observation points to a role for differentially expressed glycoelements as ligands for tissue receptors. In principle, endogenous lectins may be able to complete a glycobiological interplay *in situ* with appropriate ligands [10–12]. Assuming a potential physiological relevance for a defined carbohydrate structure, the next step will be to determine whether this glycomolecule exhibits ligand properties for specific binding sites in the tissue. By conjugation of the carbohydrate(s) of interest to a labelled, histochemically inert carrier, a suitable probe for the search for receptor sites is engineered, termed a neoglycoconjugate [13–16]. The application of neoglycoconjugates has already been shown to be feasible for routinely available tissue material [17, 18]. Since the carbohydrate part is employed to detect specific binding sites like lectins in the tissue in contrast to the more common lectin histochemistry for glycoconjugate localization, this approach is referred to as

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'reverse lectin histochemistry' [19] or by the more general term 'ligandohistochemistry'. The combination of custom-made chemical oligosaccharide synthesis with histopathological analysis will enable study of the extent of expression of sugar-binding sites that are accessible and not adversely affected by tissue processing.

A prominent place among the tumour-associated carbohydrate antigens is taken by the blood group antigens [9, 20–22]. Besides describing the regulation of their expression recent studies implicate such carbohydrate epitopes in cell adhesion and cell mobility control [23, 24]. A tetrasaccharide that is structurally related to the blood group antigens A and Le^x inhibits proliferation of normal and transformed neural cells *in vitro* and carrier-immobilized lacto-*N*-fucopentaose induces cytokine secretion by spleen cells [25, 26]. These indications for a functional involvement are substantiated by the current effort to correlate reliably the presence of distinct blood group antigen-related oligosaccharide structures to prognosis in lung cancer [27–31]. Implying mediation of a response by binding of these oligosaccharides to specific sites, systematic assessment of the presence of blood group antigen-binding receptors in lung cancer is warranted.

Neoglycoproteins with a rather simple carbohydrate structure have already been employed to detect the presence of sugar receptors like lectins in lung cancer [32–36]. Besides a protein as carrier an artificial polymer, namely *N*-substituted polyacrylamide, can be used [19, 37, 38]. This paper presents further studies to determine whether specific sugar-binding sites are present in lung cancer and whether cell type-associated differences are apparent for a panel of neoglycoconjugates. In addition to an array of blood group antigen-related structures (Table 1), we include a galactose-3-sulfate-bearing neoglycoconjugate. This modified sugar has been implicated in cell adhesion [39–41]. The biotinylated lectin from UDA with its specificity for *N,N'*-diacetylchitobiose serves as a control for the concomitant present of glycosylation of the tumour cells.

Materials and methods

Synthesis of neoglycoconjugates

The blood group antigen-related di- and trisaccharide derivatives and the 3'-sulfated galactose derivative were synthesized as described in detail elsewhere [42–45]. The aminopropyl glycosides and a C_6-spacered trifluoroacetate derivative of biotin were attached to poly(4-nitrophenylacrylate), obtained by polymerization of 4-nitrophenylacrylate in the presence of azodiisobutyronitrile, to reach a 20% and 5% molar content, respectively, in the carrier with an overall molecular weight of approximately 40 kDa [46, 47]. Ethanolamine was used to block residual activated sites.

Table 1. Carbohydrate structures of the biotinylated neoglycoconjugates.

Abbreviation	*Structure*
Le^c(di)	Galβ1-3GlcNAcβ1-R GlcNAcβ1-R
F3G	3 | Fucα1
	GlcNAcβ1-R
F4G	4 | Fucα1
	Galβ1-4GlcNAcβ1-R
Le^x	3 | Fucα1
	Galβ1-R
H(di)	2 | Fucα1
	Galβ1-3GlcNAcβ1-R
H(I, tri; Le^d)	2 | Fucα1
	Galβ1-3GalNAcα1-R
H(III, tri)	2 | Fucα1
A(di)	GalNAcα1-3Galβ1-R
	GalNAcα1-3Galβ1-R
A(tri)	2 | Fucα1
Gal-3-SO_4	HSO_4-3-Galβ1-R

Tissue preparation

The tissue specimens originated from 121 patients who underwent surgery for lung cancer at the Thoraxklinik in Heidelberg in the period of 1992–1993. The tumour cases are divided into 29 epidermoid carcinomas, 25 adenocarcinomas, 20 large cell carcinomas, 17 small cell carcinomas, 19 mesotheliomas and 11 intrapulmonary metastases of extrapulmonary primary carcinomas. The classification of the tumour cell type was based on the common WHO rules and post-surgical TNM-staging followed the UICC rules [36]. At least one complete tumour cross-section for each case was analysed. The surgical specimens had been insufflated with buffered formalin via the main bronchi and then fixed for a minimum of 24 h prior to embedding into paraffin. The routine marker analysis included the HE, PAS, Feulgen and modified Sirius staining reactions and in the case of mesotheliomas application of probes that visualize CEA, vimentin, keratin as well as lysoganglioside GM_1- and *N*-acetylglucosamine-specific binding sites.

Reverse lectin histochemistry

Serial sections of each specimen (4–5 µm thickness) were treated under identical conditions. They were soaked in

xylene to remove paraffin and rehydrated in graded alcohol solutions. The endogenous peroxidase was blocked by incubation with 1.5% methanolic hydrogen peroxide for 30 min. Endogenous biotin that would react with the ABC kit reagents was saturated by application of a commercially available blocking kit (Camon, Wiesbaden, FRG). The biotinylated markers were incubated at a concentration of 10 $\mu g\ ml^{-1}$ in phosphate-buffered saline for 60 min at room temperature on the pre-treated sections. Commercial ABC kit reagents and the chromogenic substrates diaminobenzidine/hydrogen peroxide were then successively applied to visualize the bound probe. The different stages of processing were separated by a thorough washing procedure to remove completely any reagent from the previous step. Finally, counterstaining with Haematoxylin was performed and the sections were mounted. The histochemical reaction was considered positive if all of the cells or clusters of cells showed dark brown staining while concomitant controls revealed no or negligible deposition of the chromogenic product. Control reactions included inhibition with respective saccharides and omission of the biotinylated probe to reveal any kit reagent-dependent binding. Differences in the extent of binding between the various probes can be considered as an additional inherent control because the chemical characteristics of the neoglycoconjugates were identical with the sole exception of the sugar moieties. The *N,N'*-diacetylchitobiose-binding lectin from UDA, purified and biotinylated as described [48, 49], was used within this protocol as an indicator for tumour cell glycosylation. Statistical analysis was performed with the *t*-test.

Results and discussion

The chemical property of the carrier part of the neoglycoconjugates and the extent of carbohydrate and label incorporation into the poly(4-nitrophenylacrylate) backbone are intentionally kept constant. Thus, the sugar part is the only structural parameter in the array of test substances that is deliberately varied. To test the influence of these structural modifications on extent of tissue binding with blood group antigen-related epitopes, nine AH- and Le-related structures have been synthesized, as compiled in Table 1. The capacity of binding of these probes, of a galactose-3-sulfate-bearing neoglycoconjugate and of the plant lectin were determined for tumour specimens from 121 patients, suffering from non-small cell or small cell lung carcinoma, mesothelioma or metastatic lesions that originated from extrapulmonary primary carcinomas (Table 2). As a further characterization of the specimens the pT- and pN-stages of the lung cancer cases are given in Table 3. In agreement with a previous study that used neoglycoproteins [32] no statistically significant correlations can be discerned between these two tumour characteristics and the number of positive cases in each group, which is summarized in Table 4.

Table 2. Characterization of the analysed tumour tissue specimens according to cell type, sex and age.

Cell type	*Male*	*Female*	*Age (years ± SD)*
Epidermoid	24	5	61.5 ± 10.6
Adeno	18	7	57.1 ± 9.4
Large cell	17	3	57.7 ± 10.8
Small cell	10	7	60.6 ± 10.2
Mesothelioma	16	3	57.9 ± 9.7
Metastasis	5	6	54.1 ± 16.0

Table 3. Characterization of the lung cancer cases according to postsurgical staging.

pT	*pN*			
	0	*1*	*2*	*3*
1	10	5	6	2
2	10	19	7	4
3	4	7	8	1
4	1	0	6	1

The marker analysis has been performed with serial sections from identically treated tissue specimens, using a constant concentration of probe, constant incubation time and kit reagents from the same lot to ensure reliable comparability. Non-uniform staining patterns are seen when the ligand part of the neoglycoconjugate or the tumour cell type are changed (Table 4). Fucosylated albumin has been described as binding less well to tissue sections from small cell lung cancer cases than from non-small cell cancer cases [32, 34]. The disaccharides F3G and F4G, but not the H-like disaccharide, show a similar relation of binding between these two tumour clases (Table 4). Notably, the A-like disaccharide (not the A-like trisaccharide) and galactose-3-sulfate exhibit no detectable binding to sections of the tested small cell lung cancer cases.

To distinguish mesothelioma from metastatic adenocarcinoma, lysoganglioside GM_1- and *N*-acetylglucosamine-exposing albumin have a similar level of accuracy as immunohistochemical tools [32, 35, 36]. Addition of a fucose moiety to *N*-acetyl-glucosamine apparently reduces or abolishes this capacity (Table 4). Le^c-disaccharide appears to have the highest level of potency to separate these two tumour types.

Inhibition or binding studies with purified mammalian galactoside-specific lectins have substantiated that subtle structural differences can significantly affect the ligand properties of a carbohydrate structure [50–58]. Lung

Table 4. Presence of binding sites for the applied biotinylated markers in the various lung tumours, given as percentage of positive cases.

Type of market	*Cancer cell type*						
	Epidermoid ($n = 29$)	*Adeno* ($n = 25$)	*Large cell* ($n = 20$)	*Small cell* ($n = 17$)	*Mesothelioma* ($n = 19$)	*Metastasis* ($n = 11$)	*Total* ($n = 121$)
Le^c(di)	3	15	60	12	72	9	27
F3G	83	42	50	29	50	46	53
F4G	55	65	40	18	22	46	44
Le^x	41	42	75	59	72	55	55
H(di)	59	62	75	70	78	63	67
H(I. tri; Le^d)	69	81	70	88	61	82	74
H(III. tri)	35	89	70	29	61	63	58
A(di)	13	42	80	0	28	36	33
A(tri)	41	62	45	47	61	55	51
Gal-3-SO_4	10	46	30	0	22	18	22
UDA	17	77	75	18	56	82	51

tumour tissue can express various lectins with the same nominal specificity for galactose [59–61]. Thus, tumour cell type-associated differences in display of individual receptor types can reasonably explain the probe-specific alterations in cell binding (Table 4). The Le^c-disaccharide and the A-like disaccharide for example bind to the various cell types with significant differences in the percentage of positive cases. Notably, introduction of a fucose moiety to yield the A-like trisaccharide markedly affects the frequency of staining of tumour sections (Table 4). A substantial increase is seen for epidermoid and small cell carcinomas and mesotheliomas, whereas the number of positive cases decreases for large cell carcinomas. The notion of a typical receptor display for individual cell types is reinforced by differential binding of the H(type I)- and H(type III)-like trisaccharides (Table 4). Immunohistochemical studies for relevant lectins, whose presence in normal lung and certain tumours has been biochemically documented [51, 59–61], are required to pursue this line of research. Investigations of three endogenous β-galactoside-specific lectins on breast cancer sections and animal tumours strongly support the above conclusion [62, 63].

Mammalian β-galactoside-binding lectins have been shown to bind well to both A- and H-like structures [50, 51, 57]. General statistical analysis of co-expression of binding sites for two marker types in serial sections of a tumour case reveals the combination of these two probes (Table 5). This analysis can thus indicate whether related carbohydrate structures, shown to bind certain lectins *in vitro*, may be recognized by a similar set of receptors in serial sections of tumour specimens. Binding of the galactose-3-sulfate-bearing probe correlates with the ligand capacity of these neoglycoconjugates but not of a Le^x-carrying marker (Table 5). Similar to the observations on binding of carbohydrate-poly(4-nitrophenylacrylate) conjugates to human haemopoietic and lymphoid cells [37], introduction of structural variations, e.g. between F3G and F4G, affects the ligand properties of the individual probes (Table 5). The lack of co-expression in statistical analysis serves as an inherent specificity control.

Table 5. Correlation of expression of binding sites for two individual biotinylated markers in the lung cancer specimens at three levels of statistical significance[a].

$p \leqslant 0.05$	$p \leqslant 0.01$	$p \leqslant 0.001$
F4G-A(tri)	Le^c-UDA	F4G-UDA
Le^x-UDA	F4G-Le^c	H(di)-H(I, tri)
H(di)-Le^x	F4G-H(I, tri)	H(di)-A(di)
H(III, tri)-F4G	Le^x-Le^c	H(di)-A(tri)
H(III, tri)-H(I, tri)	H(di)-F3G	H(I, tri)-A(tri)
A(di)-A(tri)	H(di)-UDA	H(III, tri)-UDA
A(tri)-UDA	H(III, tri)-A(di)	A(di)-UDA
Gal-3-SO_4-H(I, tri)	H(III, tri)-gal-3-SO_4	Gal-3-SO_4-H(di)
	Gal-3-SO_4-F3G	Gal-3-SO_4-A(di)
	Gal-3-SO_4-UDA	Gal-3-SO_4-A(tri)

[a] Abbreviations are given in Table 1.

In conclusion, systematic variations of the sugar structure in combination with histo-pathological analysis are warranted in order to devise glycohistochemical tool with favourable diagnostic properties. The results of this study indicate that cell type-associated differences in the capacity to bind carbohydrate ligands can occur in lung cancer, as for example observed with the Le^c-disaccharide between mesotheliomas and metastatic adenocarcinomas or with an A-like disaccharide, fucose-α1-3/4-*N*-acetylglucosamine-β1-R or 3′-sulfated galactose between small cell and non-small cell carcinoma cases. The combination of chemical and

histopathological as well as biochemical and immunohistochemical approaches may eventually lead to a neoglycoconjugate with a carbohydrate ligand of appropriate selectivity.

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