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Differential binding activities of lectins and neoglycoproteins in human testicular tumors

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Abstract Testicular germ-cell tumors, a morphologically and clinically diverse group of malignancies provide an ideal model for investigating the biology of glycoconjugates because the biosynthesis of oligosaccharide chains of glycoproteins monitored by plant/invertebrate lectins often changes during tumorigenesis, tumor progression, and metastasis. To investigate such changes in germ-cell tumors, we analyzed 67 surgical specimens from 31 seminomas, 32 embryonic carcinomas, and four choriocarcinomas using glyco- and immunohistochemistry that involved five plant/invertebrate lectins, 16 neoglycoproteins, and galectin-1 antibody. The results showed that some of these markers, such as melibiose-, lactose-, and β-N-acetylgalactosamine-BSA-biotin were clearly differentially expressed amongst these tumors and between primary and metastatic embryonic carcinomas. The differences in staining for positivity, intensity, and heterogeneity indicate that the differential display of glycoconjugates in tumor cells may be important in tumor growth, metastasis, or prognosis because subtypes of these tumors behave quite differently from one another. Furthermore, we also found identical staining for positivity between most neoglycoproteins and their corresponding lectins, though the staining intensity of neoglycoproteins was weaker. This suggests that neoglycoproteins may be useful markers to replace their plant lectins.

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Introduction

Testicular germ-cell tumors including seminoma, embryonic carcinoma, and choriocarcinoma are the most common solid tumors in men between the ages of 20 and 40 years. Their worldwide incidence rate has more than doubled during the past four decades [3]. These tumors exhibit quite diverse morphology, clinical outcome and prognosis [3, 51]. Seminoma, for instance, is a relatively benign tumor with a favorable outcome, whereas choriocarcinoma is aggressive and has an adverse prognosis [51]. Representing a spectrum of malignancies, these tumors provide an ideal model for investigating the glycobiology in tumorigenesis and tumor progression. Though commercial plant lectins have already been used for this purpose [21, 23, 30], the presence of endogenous lectins in these tumors [12, 15] does encourage a new approach to assess binding capacities for carbohydrates in situ.

Cell-surface glycoconjugates are known to play an important role in such cell-to-cell interactions as recognition, communication, and adhesion [4, 21, 23, 29, 30, 34, 45, 46, 47, 54]. Such interactions are also important in tumorigenesis, tumor progression and metastasis [18, 27, 34, 44, 45]. During cell differentiation [29, 41] and malignant transformation [10, 24], the biosynthesis of oligosaccharide chains of glycoproteins is often altered and the alterations can be detected with lectins [7, 8, 9, 24, 35, 46, 47, 52], which are carbohydrate-binding proteins found widely in plant and animal tissues [23, 27, 54]. Their specific carbohydrate-binding properties allow lectins to recognize particular glycoconjugates. This suggests that lectins participate in the regulation of physiologic processes that involve binding or cross-linking of specific glycoconjugates [6, 54]. Galectin-1, for instance, is involved in growth

regulation and induction of differentiation in human neuroblastoma cells [31]. Neoglycoconjugates, carrier-immobilized carbohydrate structures, are also suitable tools for such detection. [5, 20, 48, 53] Therefore, we analyzed the binding sites of plant/invertebrate lectins, neoglycoproteins, and galectin-1 in surgical specimens from testicular cancer patients by using glyco- and immunohistochemistry to evaluate glycohistochemical properties of lectins and glycoconjugates in testicular germ-cell tumors.

Materials and methods

Tissue samples

Surgical specimens from 67 cases of human testicular germ-cell tumors were obtained from the Institute of Pathology, University of Göttingen, Germany. Of these, 31 cases were seminoma (the mean age of patients was 39 years, range 21–81 years), 32 cases were embryonic carcinoma (the patients' mean age was 25.5 years, range 16–39 years); and four cases were choriocarcinoma (the patients' ages were unknown). These samples were routinely fixed in 10% buffered formalin and embedded in paraffin. All specimens were cut into 4 μm-thick sections and stained with hematoxylin and eosin for classification.

Lectins and neoglycoproteins

The following plant/invertebrate lectins were used in the study: Concanavalin A (ConA)-biotin, *Dolichos biflorus* agglutinin (DBA)-biotin, *Helix pomatia* agglutinin (HPA)-biotin, *Ulex europaeus* agglutinin (UEA-1)-biotin, and *Viscum album* agglutinin (VAA)-biotin (Sigma Chemicals Co., St. Louis, Mo.). A polyclonal antibody against human galectin-1 was obtained and rigorously checked for specificity, as described elsewhere [22]. Lactose (lac)-(diaz)-bovine serum albumin (BSA)-biotin, asialofetuin (ASF)-biotin, melibiose (mel)-(diaz)-BSA-biotin, α-*N*-acetylgalactosamine (α-galNAc)-(diaz)-BSA-biotin, β-*N*-acetylgalactosamine (β-galNAc)-(diaz)-BSA-biotin, β-*N*-acetylglucosamine (β-glcNAc)-(diaz)-BSA-biotin, mannose (man)-(diaz)-BSA-biotin, L-fucose (fuc)-(diaz)-BSA-biotin, L-fucose-(thion)-BSA-biotin, maltose (mal)-(diaz)-BSA-biotin, xylose (xyl)-(diaz)-BSA-biotin, sialic acid (Neu5Ac)-(diaz)-BSA-biotin, sialic acid-(epichl)-BSA-biotin, glucuronic acid (glcA)-(diaz)-BSA-biotin, heparin-x-biotin and fucoidan-x-biotin were synthesized according to routine methods, using conjugation of *p*-aminophenyl derivatives such as diazomium ions (diaz) or *p*-isothiocyanate forms (thion) or of (2,3-epoxypropane)-4-oxybutyric acid derivatives of a monosaccharide via carbodimide mediation (epichl) for neoglycoproteins and mild cyanogen bromide treatment for the sulfated glycans (heparin, fucoidan) [1, 2, 13, 42]. The coupling efficiency was determined with 9–12, 24–28, and 20–24 moieties, respectively. The neoglycoproteins were then finally biotinylated via residual amino groups of the carrier protein to incorporation of 10–14 label units as described [13, 19].

Glyco- and immunohistochemical staining

Specific binding sites for the plant lectins, neoglycoproteins, and galectin-1 were localized in tissue sections using glyco- and immunohistochemical staining of the avidin-biotin complex (ABC) technique [26]. Briefly, the sections were deparaffinized in xylene and rehydrated in serial ethanols (100–50%). Putative endogenous peroxidase activity was inhibited by incubating tissue sections in 1% methanolic hydrogen peroxide for 30 min. Nonspecific binding of the secondary antibody for galectin-1 was blocked by preincubation with 20% normal goat serum. To minimize the nonspecific binding of BSA-biotin derivatives by protein-protein interaction, the sections were incubated with 0.1% BSA for 30 min to block protein-binding sites. The sections were then incubated at 23 °C for 4 h with the lectins, neoglycoproteins, or anti-galectin-1 antibody in 0.1% BSA/phosphate-buffered saline (PBS) solution (12.5 to 50 μg/ml for lectins, 100 to 200 μg/ml for neoglycoproteins, and 3 μg/ml for anti-galectin-1). After the sections were washed three times in PBS, they were incubated for 30 min at 23 °C with biotinylated goat anti-rabbit immunoglobulin G (IgG H + L) (Vector Laboratories, Burlingame, Calif.) for galectin-1 detection. Sections for the rest of the markers were incubated with avidin-biotinylated peroxidase complex (ABC-kit, Vector, Burlingame, CA, USA) for 30 min in the dark, followed by incubation in 3-amino-9-ethylcarbazole/hydrogen peroxide solution (Sigma) for 10 to 15 min to visualize the bound antibody by the colored peroxidase reaction product. The sections were then mounted with coverslips in glycerin/gelatine and examined with a microscope.

Control sections

To ascertain the specificity of neoglycoprotein binding, the control sections were preincubated with homologous nonbiotinylated carbohydrate-BSA-biotin conjugates for 2 h to mask sugar-specific receptors and then incubated for a further 4 h with a mixture of corresponding unlabeled and labeled neoglycoproteins in a ratio of 1:100 at concentrations of 100–200 μg/ml. A control without any labeled neoglycoprotein was also performed to exclude any possible background from ABC-kit reagents. In addition, a control with BSA-biotin was also performed to exclude any non-specific binding.

For lectin staining, the control sections were preincubated with a nonlabeled corresponding sugar (Table 1) for 2 h and then further incubated with a mixture (in a ratio of 1:100) of corresponding sugar and lectin for 4 h.

Review and scoring of sections

The stained sections were examined with a Zeiss microscope by two independent pathologists. The staining of the sections was assigned a score ranging from 0 to 3, with 0 representing no staining, 1+ weak positive, 2+ positive, and 3+ strongly positive. Staining heterogeneity was measured as a score also, ranging from 0 to 3, with 0, representing no staining, 1+ less than 50% of tumor cells positive, 2+ 50–80% positive, or 3+ more than 80% positive. Staining intensity and heterogeneity indices represented the average staining intensity or heterogeneity, respectively. Statistical analysis was performed using Pearson's chi-square or McNemmar test, respectively.

Table 1 Sugar binding specificity and specific inhibitory sugar of lectins [23]

Lectin	Origin	Carbohydrate binding specificity	Specific inhibitory sugar
ConA	Conavalia ensiformis	α-man > α-glc > α-glcNAc	α-man
DBA	Dolichos biflorus	D-galNAc	D-galNAc
HPA	Helix pomatia	α-galNAc > α-glcNAc >> α-gal	D-galNAc
VAA	Viscum album	D-gal	D-gal
UEA-1	Ulex europaeus	L-fuc	L-fucose

Results

Seminoma

Histochemical staining of 31 seminoma using 16 biotinylated neoglycoproteins, five plant/invertebrate lectins, and galectin-1 antibody, showed a high positive staining in lac-, xyl-, fuc-, and α-gal*N*Ac-(diaz)-BSA-biotin, ASF-biotin, ConA, and UEA-1, while mel-, β-gal*N*Ac, β-glc*N*Ac, man-, mal-, Neu5Ac(diaz)-BSA, Neu5Ac(epichl)-BSA, galectin-1, VAA, and DBA showed a moderate affinity to the tumor cells. The remaining five sugar-specific probes [fuc(thion)-BSA, glcA(diaz)-BSA, heparin-X, fucoidan-X, and HPA] showed only a weak affinity to these tumor cells (Table 2 and Fig. 1). The binding was quite specific and a non-specific interaction of negatively charged probes to the tissue sections can be excluded, because glucuronic acid failed to bind to *N*-acetylneuraminic acid and even a single difference in saccharide structure can result in different binding activities.

Most of the tumor cells showed heterogeneous binding to the lectins and neoglycoproteins (Table 2). Strong heterogeneity was detected in mel-, glcA-, Neu5Ac-, β-glc*N*Ac-(diaz)-BSA-biotin, fuc(thion)-BSA-biotin, heparin, and fucoidan-biotin. Lac-, man-, xyl(diaz)-BSA-biotin, ASF-biotin, VAA, ConAUEA-1 exhibited low heterogeneity compared with α-gal*N*Ac-, β-gal*N*Ac-, fuc-, mal(diaz)-BSA-biotin, Neu5Ac(epichl)-BSA-biotin, anti-galectin 1, HPA, and DBA, which showed moderate staining heterogeneity.

In comparing the staining patterns of exogenous lectins with neoglycoproteins bearing a lectin-reactive sugar, we found only one statistical difference in the positivity between HPA and α-gal*N*Ac(diaz)-BSA-biotin ($P < 0.05$). The staining intensity was, however, always stronger for lectins than for their corresponding neoglycoproteins. For the anomeric configuration of the sugar, i.e., α-gal*N*Ac and β-gal*N*Ac, no difference was found in staining positivity, intensity, and heterogeneity. In contrast, a difference was found between fuc(diaz)- and fuc(thion)-BSA ($P < 0.01$) for the coupling modes of the sugar to BSA, but no difference was detected between Neu5Ac(diaz)- and Neu5Ac(epichl)-BSA-biotin.

Embryonic carcinoma

Compared with seminoma, embryonic carcinoma is more common in younger patients and its prognosis is usually unfavorable. Stained with the 21 lectins, neoglycoproteins, and galectin-1, this tumor exhibited a high positivity only in fuc(diaz)-, xyl-(diaz)-BSA-biotin, ASF-biotin, VAA, and ConA. The lac-, α-gal*N*Ac, β-glc*N*Ac-, man-, mal(diaz)-, Neu5Ac(epichl)-BSA-biotin, galectin-1, and UEA-1 produced moderate positivity, while positivity was low in the remaining lectins and neoglycoproteins (Table 2). The staining pattern of tumor cells included focal or diffuse distribution of positive signals in the cytoplasm, and sometimes on the cell membrane (Fig. 1).

The tumor cells also showed some staining heterogeneity; for instance, mel-, β-glc*N*Ac, Neu5Ac-, glcA(diaz)-BSA-biotin, fuc(thion)-BSA-biotin, HPA, and DBA showed strong heterogeneity, while lac-, α-gal*N*Ac-, β-gal*N*Ac-, man-, mal-, xyl-(diaz)-BSA, Neu5Ac(epichl)-BSA-biotin, ASF-biotin, and galectin-1 a moderate heterogeneity compared to the low hetero-

Table 2 Differential expression, staining intensity, and staining heterogeneity of testicular germ-cell tumors with lectins and neoglycoproteins

Probes	Seminoma			Embryonic carcinoma			Choriocarcinoma		
	%[a]	II[b]	HI[c]	%	II	HI	%	II	HI
lac	89.6‡	2.15	2.00	75.0	1.57	1.62	50.0‡	1.50	1.50
ASF	89.6	2.04	2.15	85.7	1.75	1.87	nd		
mel	58.6†	1.18	1.18	25.0†	1.00	1.00	25.0	1.00	1.00
α-galNAc	82.7#	1.63	1.83	71.4	1.40	1.55	50.0	1.50	1.50
β-galNAc	65.5‡	1.63	1.79	50.0	1.21	1.57	0‡	0	0
β-glcNAc	55.1	1.38	1.31	53.5	1.00	1.13	nd		
man	75.8	1.86	2.18	60.7#	1.65	1.70	75.0	1.33	1.33
fuc(diaz)	89.6*	2.08	1.92	82.1*	1.87	2.00	75.0	1.33	1.33
fuc(thion)	6.8*	1.00	1.00	7.1*	1.00	1.00	nd		
mal	75.8	1.91	1.95	67.8	1.53	1.89	nd		
xyl	89.6	2.15	2.08	82.1	1.39	1.65	nd		
Neu5Ac(diaz)	58.6	1.24	1.24	35.7	1.10	1.20	25.0	1.00	1.00
Neu5Ac(epichl)	65.5	1.47	1.74	57.1	1.25	1.50	nd		
glcA	44.8	1.08	1.38	32.1	1.00	1.00	nd		
heparin-	10.3	1.00	1.00	3.5	2.00	2.00	0	0	0
fucoidan-	10.3	1.00	1.00	3.5	2.00	3.00	nd		
anti-galectin-1	65.5#	1.42	1.79	64.2	1.41	1.64	50.0	2.00	1.50
VAA	79.3	2.17	2.04	85.7	2.17	2.29	100	2.25	3.00
HPA	34.5#	1.10	1.50	50.0	1.07	1.43			
DBA	62.1	1.22	1.72	50.0	1.00	1.29			
ConA	89.6	2.15	2.42	96.4#	1.70	2.30			
UEA-1	82.8	2.21	2.13	71.4	1.90	2.20			

[a] Percentage of positive staining
[b] Staining intensity index
[c] Staining heterogeneity index
nd Not done
*$P < 0.01$, X^2 test between fuc(diaz) and fuc(thion)-BSA-biotin
†$P < 0.05$, X^2 test between seminoma and embryonic carcinoma
‡$P < 0.05$, X^2 test between seminoma and choriocarcinoma
#$P < 0.05$, X^2 test between the plant lectin and neoglycoprotein

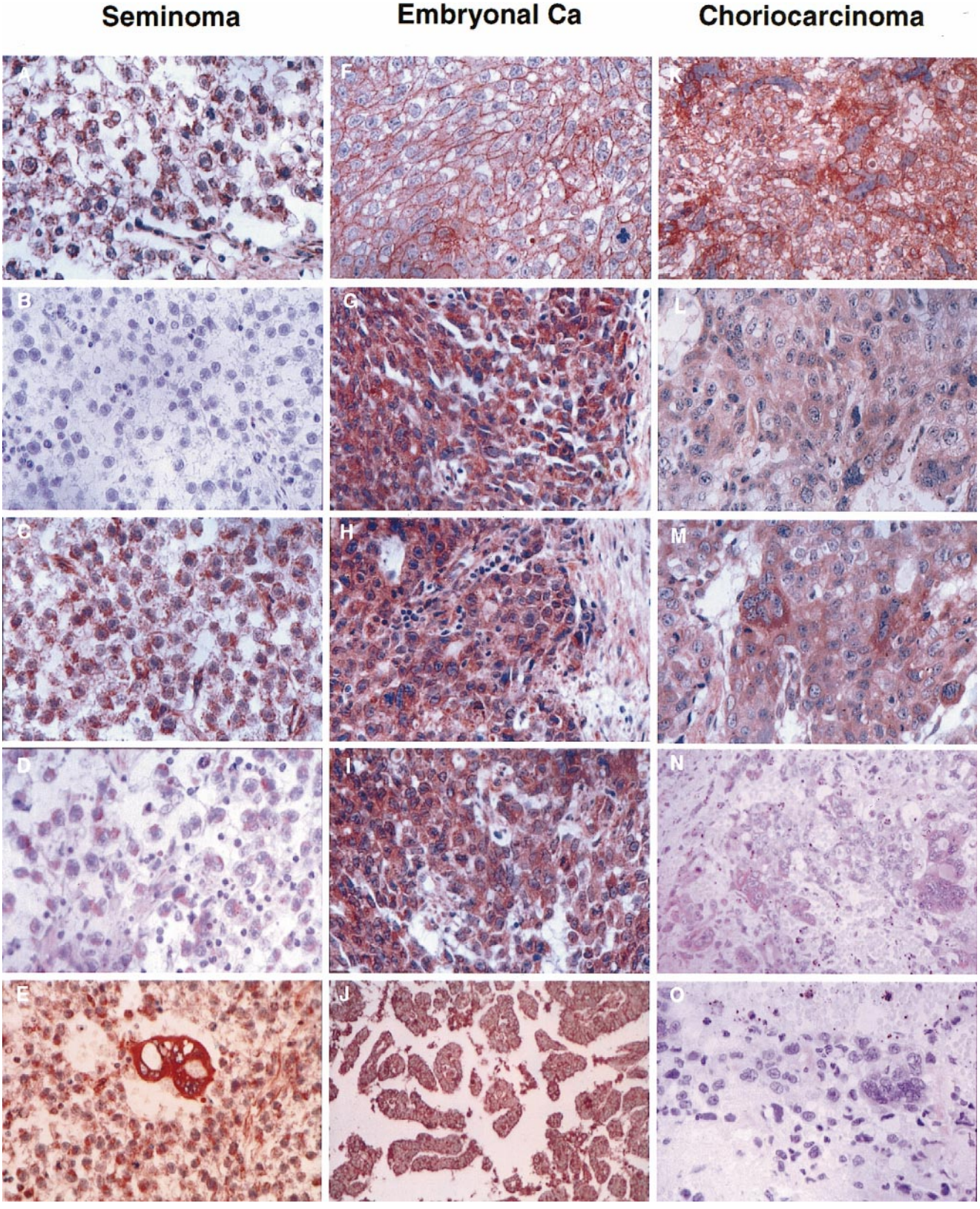

Fig. 1 Analysis of testicular germ-cell tumors with lectins, neoglycoproteins, and anti-galectin-1 antibody using glyco- and immunohistochemistry. Seminoma with (**a**) X560, fuc(diaz)-BSA-biotin (**b**) X560, fuc(thion)-BSA-biotin, (**c**) X560, UEA-1, (**d**) X560, α-galNAc(diaz)-BSA-biotin, and (**e**) X320, DBA-biotin; embryonic carcinoma with (**f**) X560 man(diaz)-BSA-biotin, (**g**) X560, lac(diaz)-BSA-biotin, (**h**) X560, UEA-1, (**i**) X560, anti-galectin-1, and (**j**) X320, ConA; choriocarcinoma with (**k**) X560, fuc(diaz)-BSA-biotin, (**l**) X560, lac(diaz)-BSA-biotin (**m**) X560, anti-galectin-1, (**n**) X320, α-galNAc(diaz)-BSA-biotin, and (**o**) X320, β-galNAc(diaz)-BSA-biotin

geneity of fuc-, mal(diaz)-BSA-biotin, heparin-X-, fucoidan-X-biotin, VAA, ConA, and UEA-1 (Table 2).

Between the lectins and their corresponding neoglycoproteins, only one difference was found in their positivity between ConA and man(diaz)-BSA-biotin ($P < 0.05$). The staining intensity of lectins, again was stronger than that of their corresponding neoglycoproteins; man-BSA-biotin had higher staining heterogeneity than ConA. For anomeric configuration of the sugar, i.e., that of α-gal*N*Ac- and β-gal*N*Ac(diaz)-BSA-biotin, no differences were detected in positivity, staining intensity, and heterogeneity. For the coupling modes, differences were found between fuc(diaz)- and fuc(thion)-BSA-biotin ($P < 0.05$), but no difference was detected between the two neoglycoproteins harboring sialic acid.

We have also analyzed metastasized tumors from nine embryonic carcinomas with these lectins and neoglycoproteins. When comparing the primary tumor with the secondary one, the results showed a great difference in staining intensity of mal(diaz)-BSA-biotin, DBA-, and UEA-1-biotin and statistical differences in positivity ($P < 0.05$, McNemmar test) of β-glcNAc- and fuc(diaz)-BSA-biotin.

Choriocarcinoma

We have only examined samples for ten lectins and neoglycoproteins for choriocarcinoma because not enough tissue sections were available for any further analysis. The assays for these ten lectin and neoglycoproteins showed, high positivity only in VAA; man-, and fuc(diaz)-BSA-biotin showed moderate positivity, while the remaining seven only yielded low positivity or no staining (Table 2 and Fig. 1). Strong staining intensity was detected only with galectin-1 and VAA-biotin; moderate staining intensity was seen in lac- and α-gal*N*Ac(diaz)-BSA-biotin; mel-, Neu5Ac(diaz)-, β-gal*N*Ac-, man-, fuc-(diaz)-BSA-biotin, and heparin-X-biotin exhibited weak or no staining (Table 2).

In terms of the anomeric configuration, we found a difference between α-gal*N*Ac- and β-gal*N*Ac(diaz)-BSA-biotin in staining positivity, but no difference was found between lectins and their corresponding neoglycoproteins, and only the staining intensity of lectins was stronger than that of their corresponding neoglycoproteins.

Differential binding activities of these markers among these tumors

Comparing seminoma to embryonic carcinoma, mel(diaz)-BSA-biotin was stained more positively in seminoma than in embryonic carcinoma ($P < 0.05$) (Table 2), and lac-, fuc-, xyl(diaz)-BSA-biotin, ASF-biotin, UEA-1, and ConA-biotin showed stronger staining intensity in seminoma than in embryonic carcinoma (Table 2). Staining for lac-, man-, xyl-(diaz)-BSA-biotin, and ASF-biotin, however, exhibited a more heterogeneous reaction in embryonic carcinoma than in seminoma (Table 2).

Comparing seminoma with choriocarcinoma, we found lac- and β-gal*N*Ac(diaz)-BSA-biotin stained more positively in seminoma than in choriocarcinoma ($P < 0.05$), while the staining intensity of lac- and fuc(diaz)-BSA-biotin was stronger in seminoma than choriocarcinoma. Anti-galectin-1 stained more weakly in seminoma than choriocarcinoma (Table 2). Lac- and man(diaz)-BSA-biotin showed more heterogeneous staining in choriocarcinoma than seminoma (Table 2). But there were no differences in positivity found between embryonic carcinoma and choriocarcinoma, although the staining intensity of fuc(diaz)-BSA-biotin was a little stronger in the former than the latter and the staining intensity of galectin-1was weaker in embryonic carcinoma than in choriocarcinoma (Table 2). No remarkable difference in staining heterogeneity was detected between these two types of tumors (Table 2).

Discussion

As far as we know, this study is the first to analyze expression of binding sites of neoglycoproteins and galectin-1 in surgical specimens of testicular germ-cell tumors, though exogenous lectins have been used widely as biomarkers in previous pathology studies. We also analyzed the staining of five plant/invertebrate lectins to compare their staining characteristics with those of corresponding neoglycoproteins. Some of the markers, e.g., mel-, lac-, and β-gal*N*Ac-(diaz)-BSA-biotin, clearly showed differential expression of binding sites in these tumors. The differences in staining positivity, intensity, and heterogeneity may indicate that the glycoconjugates in tumor cells are important in tumorigenesis, tumor progression, or metastasis because subtypes of these tumors behave quite differently. Thus, these lectins, especially neoglycoproteins may offer a perspective for further functional study of testicular tumors.

Glycoconjugates, major constituents of the cell membrane, contain biological information relevant to cell recognition, growth, and differentiation. Thus detection of cell differentiation or transformation based on changes in the sugar constituents of glycoconjugates has been found to be a useful measure in modern pathology [8, 9, 19, 52]; sugar chains of glycoconjugates exert their functional roles, at least in part, by specific recognitive interactions with endogenous carbohydrate-binding proteins such as lectins [18, 21, 28]. Thus, the histochemical localization of these glycoconjugates with lectin or neoglycoprotein may provide us with biomarkers for tumor characterization and probably for tumor classification as well [1, 8, 9, 52].

Several studies have demonstrated the presence of plant lectin-binding glycoconjugates in germ cells and its tumors [17, 25, 32, 33, 36, 37, 38, 39, 40, 43, 49, 50]. Several investigators have even suggested the value of

certain lectins in the diagnosis of testicular germ-cell tumors, though some results were not conclusive [25, 32, 33, 36, 37, 38, 39, 40, 43, 49, 50]. So far no study has analyzed the binding sites of neoglycoproteins in testicular germ-cell tumors. By analyzing 67 surgical specimens from these tumors, we found differences in staining positivity, intensity, and homogeneity between these tumors and between the primary and secondary embryonic carcinomas. Significant differences in lectin- and neoglycoprotein-binding in tumor cells may indicate a biological diversity amongst these tumors and provide a basis for further investigation and ultimately a clearer understanding of these tumors.

Our data agree with those of other investigators who analyzed the binding patterns of different plant lectins (ConA, DBA, HPA, and UEA-1) in embryonic carcinoma [32, 49, 50] and partially with data found in seminoma [37, 38]. However, our data differ from those studies of DBA, HPA, and UEA-1 in seminoma [33, 39], for which a direct-fluorescence technique was used and the sensitivity of the technique was much lower than with ABC-technique [26].

Our study showed heterogeneous binding of lectins, neoglycoprotein, and galectin-1 to tumor cells. Heterogeneous expression was a common phenomenon in tumors; those with low differentiation exhibited greater heterogeneity than highly differentiated ones. The reason for heterogeneity is not clear but, in general, the heterogeneous expression of cellular antigens was not artificial – the result, for instance, of uneven fixation or staining. As Edwards [11] has demonstrated, antigenic heterogeneity of the cell surface really exists; it is not a property exclusive to tumor cells but found in a wide range of normal epithelia. He showed that antigenic heterogeneity is not related to the mitotic cell cycle but could not say whether variations of antigen expression reflect variations in the differentiated or mature state of the cell. The staining heterogeneity of tumor cells may arise from reversible variations in gene expression as well as irreversible differentiation, or from genetic changes, which may affect the ability of tumor cells to invade and seed in metastatic sites. Our group [14, 15] has also demonstrated that heterogeneity may reflect the presence of different subpopulations of tumor cells, each displaying modified sugar receptor patterns. We believe that the heterogeneity of endogenous lectin receptors in tumor cells may be related to tumor cell growth, differentiation, and the degree of malignancy or metastatic capability [16].

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