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Neoglycoprotein binding to colorectal tumour cells: comparison between primary and secondary lesions

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Summary. Biotinylated neoglycoproteins are useful to determine the expression of sugar receptors (lectins) histochemically in routinely processed tissue sections. Assessment of the presence of distinct receptor classes with specificity to β-galactosides and to α- or β-*N*-acetylgalactosamine, selected on the basis of their potential relevance for recognition processes within the metastatic cascade in murine model systems, was performed for a common human tumour type, colorectal cancer. The four different types of neoglycoproteins, derived from covalent attachment of commercially available derivatives of β-*N*-acetylgalactosamine, differed only quantitatively in their capacity to detect specific binding on cultured cells and tissue sections, thus posing no major restriction on the choice of synthetic process for histochemical efficiency of the product. Glycocytological application revealed specific probe binding and a regulation of level of receptor expression for a human colon carcinoma cell line primarily for *N*-acetylgalactosamine-specific receptors upon retinoic acid-induced differentiation. Monitoring of sections of the 12 cases of primary and secondary colorectal lesions invariably disclosed the presence of the respective receptors, the extent of cell labelling in primary tumours and metastases being similar. Establishment of metastases, even in different target organs, is apparently not followed by a major phenotypic variation in this feature.

Key words: Colon cancer – Metastasis – Lectin – Neoglycoprotein – Histochemistry

Introduction

The metastatic spread of neoplastic cells, initiated by cell detachment from the primary tumour into circulation, and their arrest and growth in target organs depends on a variety of properties of tumour cells which are still not accurately defined, and on the reactions of various types of host cells and their microenvironment (Nicolson 1988; Weiss et al. 1989). In addition to the study of murine model systems possessing inherent differences in their propensity for metastasis, the thorough histopathological profiling of the expression of deliberately selected characterisitics in tissue sections of clinically apparent tumours may provide valuable evidence in discerning correlations between the presence of certain determinants and the establishment of secondary lesions. With respect to protein-carbohydrate interactions, attention has so far been mainly focussed on the characterization of cellular glycoconjugates (Alhadeff 1989; Hakomori 1989; Walker 1989). However, the elucidation of the potential significance of the putative receptors for these carbohydrate structures also deserves experimental efforts (Monsigny et al. 1988; Raz et al. 1990; Gabius 1991). This suggestion is underscored by the interference of *N*-acetylgalactosamine with adhesion of cells from certain hepatocarcinoma and leukaemia lines to stromal cell layers or immobilized glycoproteins and the importance of presence of a β-galactoside-specific lectin for metastasis formation in murine fibrosarcoma cells (Stanford et al. 1986; Gabius and Gabius 1990; Gabius et al. 1990a; Raz et al. 1990). This line of evidence has served as the rationale to delineate the presence of receptor sites sites in primary and secondary lesions of a clinically common tumour type with high incidence, namely colorectal adenocarcinoma, using glycohistochemistry in routinely processed tissue sections. Neoglycoproteins can be prepared by various methods and so we compared the capacity of four types of neoglycoproteins to provide information on potential differences in their efficiency for lectin detection. In addition to the histochemical localization of binding sites for the ligands lactose and *N*-acetylgalactosamine in tumour sections, we have also demonstrated the influence of chemically induced differ-

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entiation on the presence of these receptors in human colon carcinoma cells in vitro. This emphasizes the modulatory effects which may act on this cellular property.

Materials and methods

In the synthesis of labelled neoglycoproteins a batch of bovine serum albumin (BSA; Biomol, Hamburg, FRG) was carefully checked to exclude the occurrence of notable non-specific binding. It was treated with periodate to abrogate any intefering activity of trace amounts of carbohydrate contamination. It was then used as carrier protein for conjugation of diazonium or *p*-isothiocyanato derivatives of *p*-aminophenyl glycosides, or for carbodiimide-mediated coupling of *N*-acetyl-D-galactosamine after its reaction to the aliphatic spacer (2,3-epoxypropane)-4-oxybutyric acid. Incorporation yields of 10 ± 2, 30 ± 2 and 22 ± 2 carbohydrate moieties per carrier molecule were achieved, as described by Gabius and Bardosi (1991). Coupling of *p*-aminophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside was also performed by using 1,2-diethoxycyclobutene-3,4-dione (squaric acid diester) as the coupling reagent, as outlined recently by Tietze et al. (1991). Briefly, a 2-fold molar excess of the reagent was added to the *p*-aminophenyl glycoside in dry ethanol and purification by silica chromatography (chloroform/methanol/water, 13/8/2) after 8 h at room temperature yielded the activated derivative with a yield of 70%, as ascertained by elementary analysis, infra-red, mass, ^{1}H- and ^{13}C-NMR spectra. Glycosylation of BSA was carried out for 3.5 h at room temperature at pH 9.0 with a 75-fold molar excess of reagent to carrier protein, affording glycoside incorporation of 14 residues per carrier molecule, as determined spectrophotometrically at 270 nm and 310 nm. The neoglycoproteins and the carrier protein as a control to ascertain absence of binding by protein-protein interaction were biotinylated with biotinyl-*N*-hydroxysuccinimide ester Sigma, Munich, (FRG).

The human colon adenocarcinoma cell line COLO 205, obtained from the American Type Culture Collection (Rockville, USA), was cultured in 90% RPMI-1640 medium and 10% fetal calf serum containing 2 mM L-glutamine and antibiotics. One of the differentiation-inducing reagents sodium butyrate (1 mM), all-*trans* retinoic acid (5 μM) and 12-*O*-tetradecanoylphorbol 13-acetate (50 ng/ml) were added as described by Gabius et al. (1990b). Cultured cells were carefully washed in Hank's balanced salt solution containing 1% BSA to remove any serum components. Cytospin preparations of 5×10^4 cells were dried. These cells, or carefully washed adherent cells that had been grown on coverslips, were fixed at 4° C in 80% acetone for 10 min and washed with buffer (20 mM HEPES with 0.9% NaCl, 20 mM $CaCl_2$ and 0.1% BSA at pH 7.5). Protein-binding sites were saturated by incubation with 2% BSA in buffer for 20 min at room temperature. After blotting off this solution the specimens were taken through a series of steps including incubation with the labelled probe (100 μg/ml), abrogation of endogenous peroxidase activity and signal development, as described by Gabius et al. (1990b). Control reactions in the absence of labelled probe, in the presence of an excess of competitive inhibitor as well as with biotinylated, carbohydrate-free carrier protein (200 μg/ml) instead of the neoglycoprotein ascertained specificity of the observed staining, were scored by two independent observers.

For glycohistochemical staining tissue specimens were obtained at autopsy and fixed in 4% formaldehyde prior to dehydration and embedding at 56° C. Cases 1–5 were primary adenocarcinomas of the large intestine; cases 6a and 7a were primary tumours with liver metastases in the same patient (6b, 7b); cases 8–10 were liver metastases from different patients; case 11 was a lung metastasis; and case 12 consisted of metastases to the liver (12a), lung (12b), peritoneum (12c), kidney (12d) and adrenal gland (12e) in the same patient. Sections (5 μm) were processed by rehydration, treatment with 1% hydrogen peroxide in methanol for 30 min, saturation of protein-binding sites, successive incubations with the labelled probe (100 μg/ml in phosphate-buffered saline at pH 7.4) and ABC reagents as well as signal development with 3-amino-9-ethyl-carbazole in conjunction with appropriate specificity controls, as described by Gabius and Bardosi (1991). The staining of the tumour cells in the individual sections was semi-quantified with respect to the cell percentage within the total tumour cell compartment as well as with respect to the intensity, and these parameters were grouped into categories.

Results

Three types of neoglycoprotein were synthesized by common protocols and we prepared another type of glycosylated carrier protein by using squaric acid diester as coupling reagent. The preparation of the activated carbohydrate derivative that was used in the conjugation step was monitored by spectrometric analyses, as documented in Fig. 1. The three types of β-*N*-acetylgalactos-

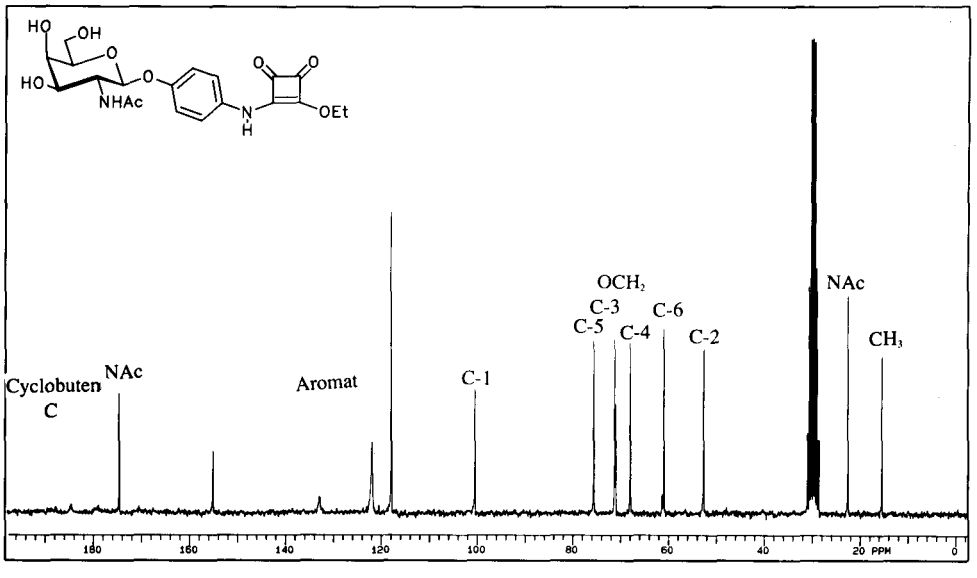

Fig. 1. ^{13}C-NMR spectrum of *p*-(4-ethoxycyclobut-1-ene-2,3-dione-1-amido)phenyl 2-acetamido-2-deoxy-β-D-galactopyranoside. Resonances of carbon atoms are denoted

Table 1. Binding of neoglycoproteins to fixed human COLO 205 colon tumour cells, treated with differentiation-inducing reagents

Chemical treatment	lac[a]		α-galNAc		β-galNAc			galNAc BSA_{22}
	BSA_{10}[d]	BSA_{30}	BSA_{10}	BSA_{30}	BSA_{10}[d]	BSA_{14}	BSA_{30}	
Without treatment	++	++	+/++	(+)/+	+	+	(+)/+	(+)/+
1 mM sodium butyrate	+/++	+/++	+	+	+	+	+	+
5 μM retinoic acid	+/++	+	+++	++	++/+++	++	++	++/+++
50 ng/ml TPA[c]	++	++	++	+/++	++	++	++	++

The staining intensity with the individual biotinylated probes and the ABC reagents is divided into four categories from (+) to +++

[a] The neoglycoprotein is designated by its carbohydrate part (lactose or *N*-acetyl-D-galactosamine) and its incorporation yield

[b] all-*trans* retinoic acid

[c] 12-*O*-Tetradecanoyl phorbol 13-acetate

[d] From Gabius et al. (1990b)

amine-containing neoglycoprotein with aromatic linkers, derived from coupling the appropriately derivatized *p*-aminophenyl glycoside, were compared in their histochemical efficiencies to neoglycoproteins that either contained the α-anomer or lactose, immobilized as diazonium derivative of the *p*-aminophenyl glycoside or *N*-acetylgalactosamine in C_6 linkage to an aliphatic spacer. These probes were used in the following studies for detection of specific binding sites in human colorectal cancer cells in vitro and in situ.

The measurements with the panel of labelled neoglycoproteins on cultured colon adenocarcinoma cells were performed to ascertain the presence of specific binding sites in vitro, the assess the efficiency of the different types of neoglycoprotein for receptor localization and to monitor the response to chemically induced differentiation. In each case, specific binding was detected (Table 1). Control reactions with carbohydrate-free biotinylated carrier protein and competitive inhibition with label-free neoglycoprotein irrespective of the type of synthesis ascertained the inherent specificity. Whereas the extent of receptor expression was similar in the case of the lactosylated marker in the absence and in the presence of the differentiation-inducing agents, a pronounced increase was noted for α-*N*-acetylgalactosamine-specific binding after treatment with retinoic acid (Table 1, Fig. 2). Only slight differences were notable with the different types of neoglycoprotein. These results encouraged us to evaluate the extent of staining of tumour cells in tissue sections semi-quantitatively.

As already described for the cultured tumour cells, control reactions were performed as illustrated in Fig. 3. The comparison of the staining intensity of the different cases revealed heterogeneity (Table 2). The α-anomer-containing labelled probe bound to the cells to a greater degree than carrier proteins with the β-anomer or lactose. The type of synthesis had no major influence on the performance of the neoglycoprotein, the labelled product from coupling by diazotization yielding the relatively highest staining intensity. A similar staining distribution was observed for primary and secondary tumours. Notably, this similarity extended to the pattern of cells of metastatic foci to different target organs (Table 2, Fig. 3).

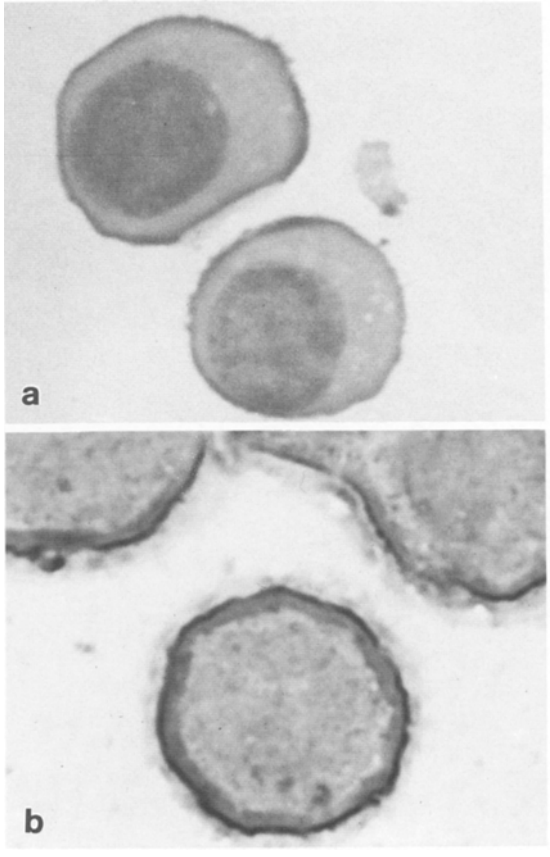

Fig. 2. Glycocytological staining of cytospin preparations of cultured human colon adenocarcinoma cells **a** without and **b** after prior treatment with retinoic acid by subsequent application of α-*N*-acetylgalactosaminylated (BSA-biotin), carrying 10 carbohydrate residues per carrier molecule, ABC reagents and the sensitive colour development system. Counterstaining was performed with **a** haemalaum and **b** Kernechtrot. ×1200

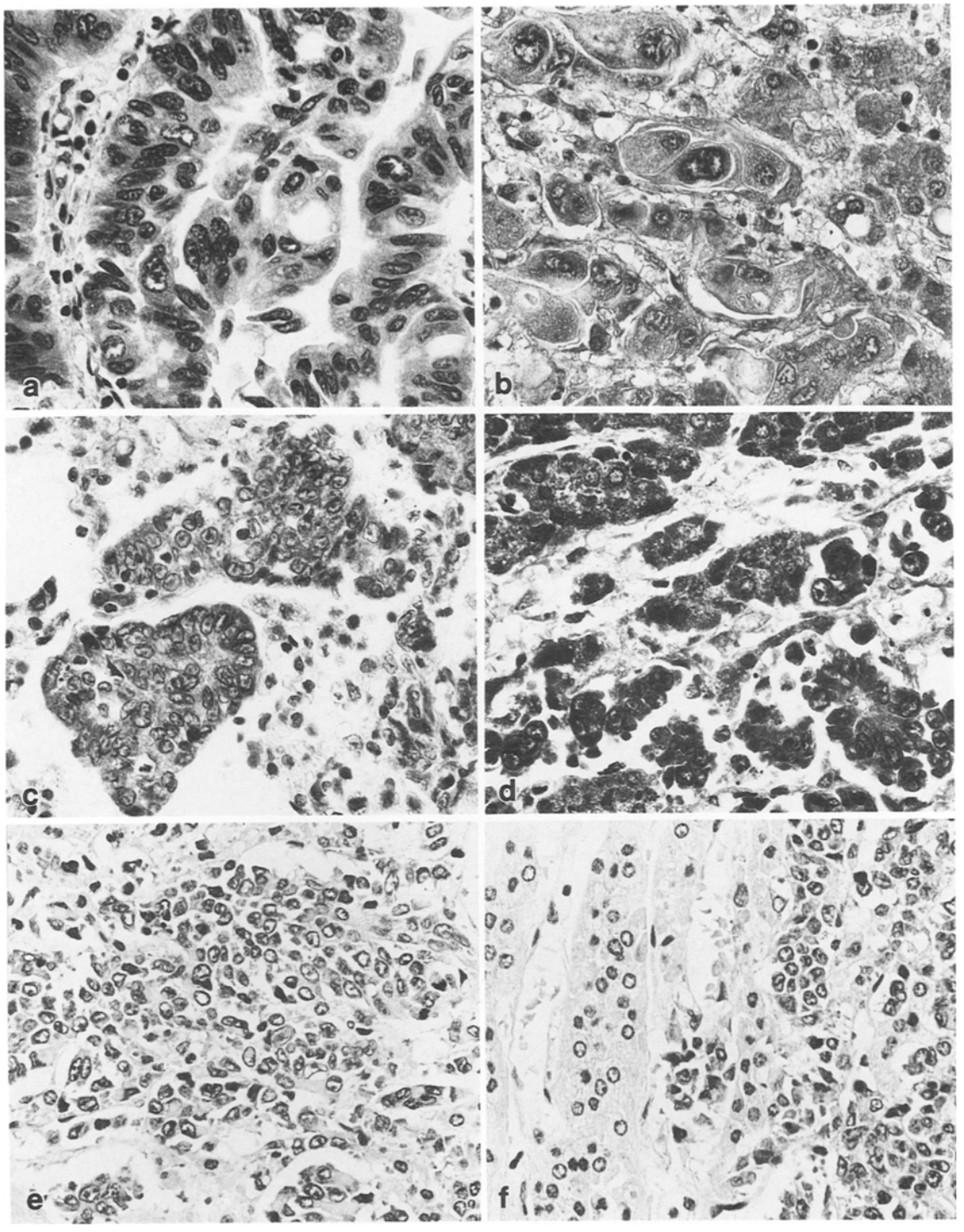

Fig. 3. Light micrographs of sections of **a** a primary adenocarcinoma of the large intestine and **b** its liver metastasis, referred to as cases 7a and 7b in Table 2, after incubation with biotinylated β-gal*N*Ac-exposing BSA_{10}. Sections of **c** a lung and **d** an adrenal cortex metastasis, referred to as cases 12b and 12e in Table 2, after incubation with biotinylated α-gal*N*Ac-exposing BSA_{10} and ABC reagents as well as after haematoxylin counterstaining. Control reactions after incubation of sections of the adrenal cortex metastasis **e** with biotinylated α-gal*N*Ac-exposing BSA_{10} in the presence of an excess of competitive inhibitor, or **f** with biotinylated carbohydrate-free carrier protein and otherwise identical treatment. $\times 375$

Discussion

The nature of the molecular properties that characterize the metastatic phenotype and that may be instrumental in determining the propensity for metastasis formation are still ill-defined. It is without question that murine model systems facilitate meaningful experiments that are designed to correlate presence of certain determinants with establishment of metastases. However, the analysis of metastasis formation in the clinical situation is re-

Table 2. Binding of neoglycoproteins to tumour cells of primary or metastatic lesions of colon carcinomas

Case	Age	P			Stage	lac-BSA_{10}	α-galNAc-BSA_{10}	β-galNAc			galNAc-BSA_{22}
		T	N	M				BSA_{10}	BSA_{14}	BSA_{30}	
1	84	4	3	1	IV	4/2	4/3	4/2	3/1	2/1	4/2
2	76	3	2	0	III	4/1	4/2	4/1	4/1	4/1	4/2
3	74	3	0	0	II	4/2	4/3–4	4/2–3	4/1–2	4/1–2	4/3
4	82	3	0	0	II	4/1	4/2	4/1	4/1–2	3/1	4/1–2
5	56	2	0	0	I	4/2–3	4/3	4/2	4/2	4/1	4/2
6a	61	4	2	1	IV	4/1	4/2	3/1	3/1	2/1	4/1
6b						4/1	4/2	4/1	4/1	2/1	4/1–2
7a	65	3	0	1	IV	4/3	4/4	4/3	4/3	4/2	4/3
7b						4/3	4/4	4/3	4/3	4/2	4/4
8	69	3	0	1	IV	4/2	4/4	4/3	4/2	4/1	4/3
9	70	4	2	1	IV	4/3	4/4	4/3	4/3	4/2	4/3–4
10	49	3	2	1	IV	4/2	4/3	4/2	3/1	4/1	4/2
11	72	4	3	1	IV	4/2	4/3	4/2	4/2	4/1	4/3
12a	46	4	3	1	IV	4/3	4/4	4/2	4/3	4/3	4/3
12b						4/2	4/3	4/2	4/1–2	4/2	4/3
12c						4/1–2	4/3–4	4/2–3	4/1–2	4/1	4/3
12d						4/2–3	4/3–4	4/2	4/2–3	4/2	4/3
12e						4/2	4/4	4/3	4/3	4/3	4/3
Average of staining						4.0/2.0	2.0/3.2	3.9/2.1	3.8/1.9	3.6/1.5	4.0/2.6

Cytoplasmic staining is evaluated by the percentage of positive cells (first value: 1, 0–5%; 2, 5–20%; 3, 20–50%; and 4, 50–100%) and by the individual staining intensity (second value: 1, weak, but significant staining; 2, medium staining; 3, strong staining; and 4, very strong staining). The neoglycoprotein is designated by its carbohydrate part and its incorporation yield

stricted to histopathological monitoring, unless other host organisms for human cancer cells like nude mice are used. Obviously, the series of events occurring during spread from an inherently heterogeneous tumour mass in the post-delivery phases of dissemination can exert modulatory influences on the phenotype, rendering clear-cut correlations more difficult. It is noteworthy that the impact of microenvironmental factors on lectin expression has already been determined in a murine model system (Glaves et al. 1989; Vidal-Vanaclocha et al. 1990). Plant lectins have proved valuable in revealing instability of defined characteristics during tumour outgrowth (Chan et al. 1985; Stanford et al. 1986). In studies with plant lectins on human colon cancer, no constant pattern of ligand expression has been disclosed (Kellokumpu 1986; Hohenberger et al. 1990). Overall, it appears reasonable to state that an unequivocal decision on definite properties of the actual metastatic phenotype presents a formidable problem. Clinically, however, assessment of features that are present in primary and secondary lesions without necessity for concurrent proof of functional importance can be helpful for therapeutic and imaging approaches (Esteban and Battifora 1990). They may include cytoplasmic epitopes (Dairkee and Hackett 1989). Based on this reasoning we profiled the expression of two purposefully chosen sugar receptor classes in tumour cells and tissue sections by biotinylated neoglycoproteins. Among the four types of methods for carbohydrate conjugation, no major influence on staining intensity following application of their products was apparent, thus posing no processing restrictions on the selection of the procedure that invariably started with commercially available derivatives. Similarly, we only included routinely processed tissue specimens to ensure continuing studies with no imposed restrictions concerning the preparation of pathological material. The in vitro study revealed a modulatory impact of retinoic acid-induced differentiation on the feature monitored. This was consistently present in a high percentage of tumour cells within the histological cases investigated. Broad phenotypic variation between malignant cells in primary and metastatic lesions on the histochemical level, defined by the tools employed, could be excluded. Since variation on the level of purified proteins with specificity to β-galactosides has been reported for xenografted tumour material of primary and secondary colon tumours (Gabius et al. 1989), refinements of the ligand structure of the neoglycoproteins by custom-made chemical synthesis is now desirable. This will enable the further characterization of the properties of the detectable binding sites in primary and secondary tumours, in normal tissue, and in benign or premalignant lesions.

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