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Wheat Germ Agglutinin Binding Sites on Human Urothelial Cells of Different Grades of Transformation

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^{125}I-Wheat germ agglutinin (WGA) binding parameters of human urothelial cell lines of different grades of transformation (TGrII and TGrIII) were compared. The values of association constant (K_a) and the number of binding sites/cell for HCV29 (TGrII) cell line were about 3 x 10^6 M^{-1} and over 4 x 10^7, respectively.Two TGrIII cell lines, HCV29T and Hu549 revealed lower values for K_a, and considerably higher numbers of binding sites/cell (about 3 x 10^8 and 2 x 10^8, respectively). Binding of ^{125}I-WGA to total cellular proteins resolved by SDS-PAGE and transferred to nitrocellulose showed multiple diffused bands in the range of 58-180 kDa. Some of these bands were characteristic for TGrII cells (124 kDa) or TGrIII cells (135 and 148 kDa).

It is well documented that such basic biological phenomena as normal cell differentiation as well as neoplastic transformation are consistently associated with temporarily ordered changes in glycosylation of glycoproteins and/or glycolipids [1-3]. The differences in oligosaccharide moieties of cell membrane glycoconjugates between normal and transformed cells and cells representing different stages of neoplastic transformation are consistently found [4-7].

Plant lectins with well defined specificities for oligosaccharide sequences are useful tools for studies on the differential expression of cell surface carbohydrates in cancer cells [7-13]. These carbohydrate-binding proteins were used by Örntoft [10] in studies on human urothelium, to evaluate the differences in cell surface oligosaccharides of normal urothelium and transitional cell carcinomas of different histological and topographical grades [10]. It was reported that the number of receptors for wheat germ agglutinin (WGA) decreased in transitional cell neoplasias with increased degree of aneuploidy [10].

Abbreviations: TGr, transformation grade; WGA, wheat germ agglutinin; sWGA, succinylated wheat germ agglutinin; GlcNAc, *N*-acetyl-D-glucosamine; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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In our previous studies on human urothelial cell lines of different grades of transformation we have found that tumorigenic and invasive cell lines showed increased staining with fluorescein-labeled WGA [14], and differ in the expression of glycoproteins and glycolipids, as compared to non-tumorigenic and non-invasive cell lines. Tumorigenic and invasive cells were characterized by the increased amounts of highly branched *N*-acetyllactosamine type glycans [15] and almost complete suppression in the expression of GM_2 ganglioside (M. Ugorski, unpublished results).

In the present investigation, we have analysed the expression of sialic acid-containing glycoconjugates on these urothelial cell lines, with the use of WGA. The binding parameters and molecular nature of the receptor for this lectin were evaluated.

Materials and Methods

Cells and Culture Conditions

The cell lines used in this investigation all originated from human urinary bladder. They were obtained from Prof. J. Kieler, The Fibiger Institute, Copenhagen, Denmark. On the basis of investigation of 19 such lines of normal as well as tumor origin, Christensen *et al.* have defined three grades of transformation (TGrI-III) [16]. As TGrII were classified cell lines with infinite life span, non-tumorigenic for nude mice and non-invasive for chick heart fragments in *in vitro* invasion tests [17]. As the TGrIII group were classified cell lines with infinite life span, tumorigenic in nude mice and invasive in *in vitro* tests. The following cell lines were used:
1). HCV29 (TGrII) - this cell line was derived from the histologically normal part of the bladder which received irradiation because of a bladder tumor [18]. 2). HCV29T (TGrIII) is a "spontaneously" transformed sub-line of the HCV29 cell line [19]. 3). Hu549 (TGrIII) - this cell line was derived from transitional cell carcinoma (TCC), which according to Bergkvist *et al.* [20] was classified as a histological grade 2 (Gr 2) tumor.

Cells were passaged using a mixture of trypsin-EDTA (Flow Labs., Irvine, U.K.) and maintained in disposable tissue culture flasks (Nunc, Roskilde, Denmark) at 37°C in 5% CO_2/95% air atmosphere in Fib 41B, which is a modified Eagles Minimal Essential Medium [21] supplemented with non-essential amino acids and 10% fetal bovine serum (both from Flow Labs.), penicillin and streptomycin (100 IU and 100 µg/ml, respectively). The cells were stored in liquid nitrogen and replaced with a fresh sample from frozen stock every two months. For all experiments, cells were harvested by mechanical means without use of proteases and suspended in PBS/0.5% BSA/0.02% NaN_3.

Lectins

Wheat germ agglutinin was purchased from Sigma (St. Louis, MO, USA). The succinylated WGA derivative (sWGA) was obtained by the method described by Monsigny *et al.* [22]. Briefly, 5.0 mg of WGA and 1.5 mg of solid succinyl anhydride (Veb Laborchemie, Apolda, G.D.R.) were added to 1.25 ml of saturated sodium acetate solution. The modification of protein was performed for 90 min on an ice-bath, and WGA was dialysed against cold water.

This procedure was repeated twice. The degree of modification was followed by free NH_2 group determination with the use of TNBS (BDH Chemicals Ltd., Poole, England). Protein determination of lectin solutions was done by the Lowry method with BSA (Sigma), as a standard.

The iodination of lectins was performed by the Iodogen method [23] using carrier free $Na^{125}I$ (Institute of Nuclear Research, Warsaw, Poland). The free iodine was removed on a Sephadex G-25 coarse column (Pharmacia, Uppsala, Sweden), which was followed by dialysis against PBS, for 48 h. The free iodine in the final preparations of ^{125}I-lectins was determined by the method of Boratynski [24]. Specific activity was 0.4×10^6 - 0.9×10^6 cpm/μg of protein for WGA and 0.8×10^6 - 1.5×10^6 cpm/μg of protein for sWGA.

Binding Assay

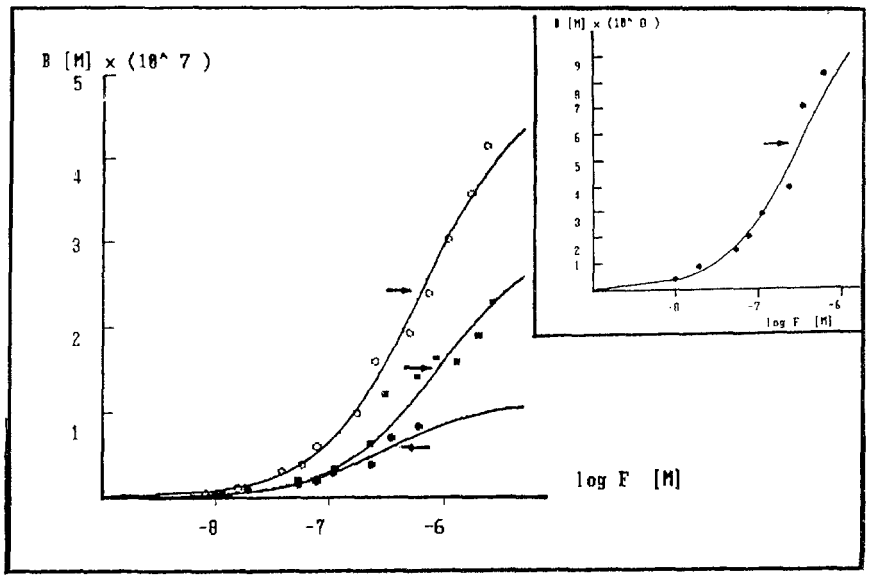
For saturation binding assay, cells were pre-incubated with an excess of unlabeled lectin (100 μg/ml, 1 h, 4°C), and specifically bound lectin was removed by incubation of the cells for 1 h at 4°C in the presence of 0.3 M *N*-acetyl-D-glucosamine (Sigma). The non-specific binding did not exceed 5% of the total binding. The assay was performed in 96-well microtiter plates (Falcon/Becton Dickinson, Heidelberg, W. Germany), pre-coated with BSA. The cells (2×10^5) were incubated in PBS/0.5% BSA/0.2% NaN_3 with increased amounts of iodinated lectin (0.014-2.8 μM), in a total volume of 0.2 ml with gentle shaking.

The competition binding assay was performed under the same conditions as was described for the saturation binding assay, except that cells were incubated with a constant concentration of ^{125}I-lectin (0.1 μM) and increasing concentrations of unlabeled lectin (2.8 - 53 μM). After incubation, cells were centrifuged through a mixture of dibutylphthalate and bis-ethylhexylphthalate (Fluka, Buchs, Switzerland), 1.1:1 by vol [25].

SDS-PAGE Electrophoresis, Blotting and Lectin Binding

Cells were dissolved in 2% sodium dodecyl sulphate, containing 2 mM phenyl-methylsulfonylfluoride (Serva, Heidelberg, W. Germany), and protein was determined according to the Lowry method. Samples containing 125 μg of protein were separated in 7.5% polyacrylamide slab gels, according to the method of Laemmli [26]. The proteins were transferred to nitrocellulose (Schleicher-Schuell BA 85, Dassel, W. Germany) [27], using a current density of 1 mA/cm^2. The non-specific binding was decreased by pre-incubating the nitrocellulose sheets with 2% gelatin solution (Loba-Chemie, Vienna, Austria) in PBS containing 0.05% Tween 20 (Serva, Heidelberg, W. Germany) for 1 h, in 40°C.

To remove sialic acid the glycoproteins bound to nitrocellulose sheets were incubated with 25 mM sulphuric acid for 1 h at 80°C. Lectin-binding glycoproteins were detected by incubating blots with ^{125}I-lectins (1×10^6 cpm/ml) for 1 h at 22°C [28]. After washing, blots were dried between cellophane sheets and subjected to autoradiography, using X-OMAT AR film for 48 h. The specificity of lectin binding to glycoproteins fixed on nitrocellulose was assessed by inhibition of the binding with 0.5 M *N*-acetylglucosamine.



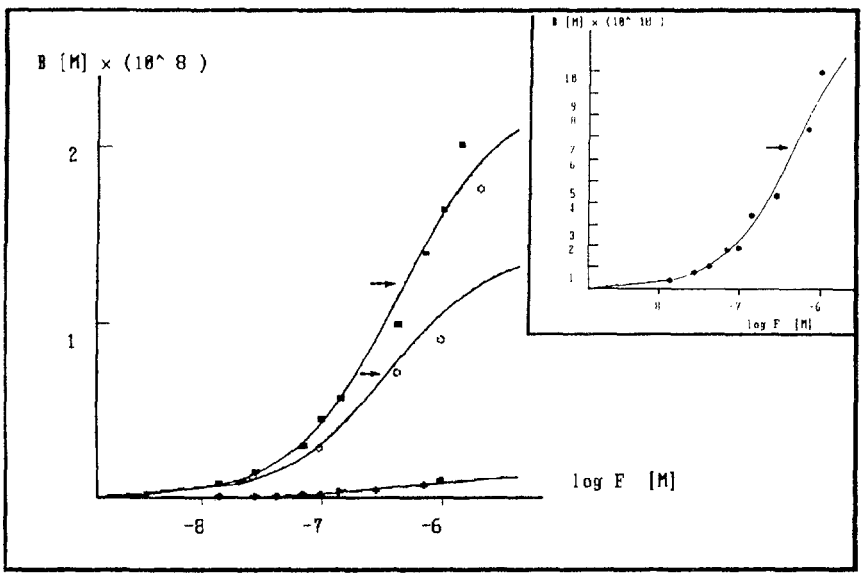


Figure 1. Representative binding curves of human urothelial cell lines [HCV29 (●), HCV29T (●) and Hu549 (■)], obtained with the use of increasing concentrations of (a) ^{125}I-WGA, and (b) ^{125}I-sWGA. Inserts: The same binding curve of (a) ^{125}I-WGA and (b) ^{125}I-sWGA to HCV29 cells, drawn on a different scale. Cells (2 x 10^5, in a total volume of 0.2 ml, in triplicate), were incubated at 4°C for 1 h; (B) = molar concentration of bound ^{125}I-lectin; (F) = molar concentration of unbound lectin. Arrows indicate the inflection point.

Results

Binding of ^{125}I-WGA and 125I-sWGA to TGrII (HCV29) and TGrIII (HCV29T and Hu549) Cell Lines

We have performed the quantitative WGA binding studies to determine the number of binding sites per cell (n) and the apparent association constant (Ka). Binding data obtained from two types of experiments (saturation binding and competition binding) were analysed by a weighted non-linear least squares curve-fitting procedure using the LIGAND program [29] with the modification of McPherson [30]. One- and two-classes of sites models were used to obtain the best fitted curve. Estimated parameters: apparent association constants (K_a) and binding capacity (B_{max}) were displayed in semi-logarithmic and Scatchard coordinates [31, 32]. Essentially the same results were obtained from the two types of experiments (saturation binding and competition binding), so, in the results, only saturation binding data are shown.

Semi-logarithmic plots of WGA binding to all analysed cell lines are shown in Fig. 1a. In the range of lectin concentrations used, the data points were available over more than half of the S-shaped curves, with values above the inflection point. Although no complete saturation was reached, still the determination of binding parameters was possible [31]. Binding of WGA was best fitted by a one-class-of-sites model for all cell lines, giving linear Scatchard plots (Fig. 2a). For TGrII cells (HCV29 cell line) the values obtained for apparent association constant and number of binding sites per cell were about $3 \times 10^6 M^{-1}$ and over 40 million, respectively. The binding of WGA to TGrIII cell lines HCV29T and Hu549 revealed lower values for apparent affinity constants, and considerably higher numbers of binding sites per cell in comparison to the HCV29 cell line (Table 1, Fig 2a). Numbers of binding sites per cell were estimated as over 300 million for HCV29T cells and almost 200 million for the Hu549 cell line (Table 1). Binding curves for sWGA binding to tested cell lines are shown in Fig. 1b. Similarly to WGA, the binding of sWGA was best fitted by the one-class-of-sites model for all analyzed cell lines, giving linear Scatchard plots (Fig. 2b). The numbers of binding sites for sWGA on cell lines HCV29 and HCV29T were about 50 times lower, and for Hu549 cell line 20 times lower as compared to those obtained for unmodified lectin (Table 1). As in the case of WGA binding, the number of binding sites per cell for the HCV29 cell line was about ten times lower than the number of binding sites found on TGrIII cell lines. The values obtained for apparent association constants were similar for all studied cell lines (Table 1). These values were higher than those obtained for WGA binding to HCV29T and Hu549 cell lines, and almost the same as in the case of HCV29 cells. In contrast to HCV29T and Hu549 cells, the variations in binding of WGA and sWGA to different batches of HCV29 cells were observed. For this reason the precision of estimated values for binding parameters for HCV29 cells, as a combination of the results from multiple experiments [29], is lower than for the remaining cell lines. However, in the particular, parallel, WGA binding experiments, the K_a values were higher, and the number of binding sites was lower for the HCV29 cell line than they were for the remaining cell lines.

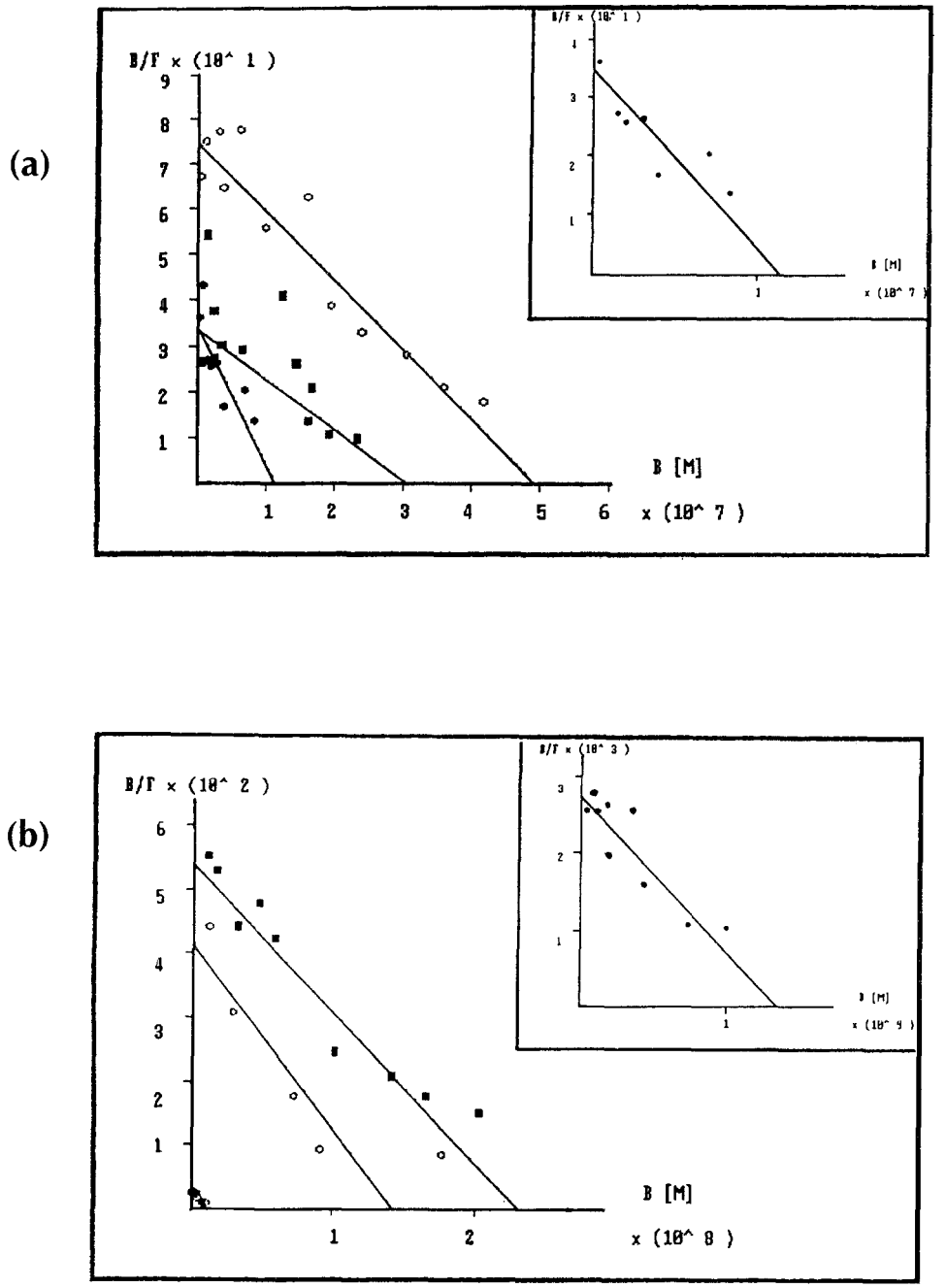


Figure 2. Representative Scatchard plots for binding of (a) ^{125}I-WGA and (b) ^{125}I-sWGA to: HCV29 (●), HCV29T (●) and Hu549 (■) cell lines. Inserts: The same Scatchard plots for binding of (a) ^{125}I-WGA and (b) ^{125}I-sWGA to HCV29 cells, drawn on a different scale. Suspensions of human urothelial cells (2 x 10^5 cells in total volume 0.2 ml, in triplicates), were incubated at 4°C for 1 h with increased concentrations of ^{125}I-lectin (saturation binding assay). (B) = molar concentration of bound ^{125}I-lectin; (F) = molar concentration of free lectin.

Table 1. Apparent association constant (K_a) and number of binding sites for ^{125}I-WGA and ^{125}I-sWGA on human urothelial cell lines[a].

Cells	^{125}I-WGA Binding		^{125}I-sWGA Binding	
	No. of binding sites/cell	K_a	No. of binding sites/cell	K_a
HCV 29	(4.19±2.02)x10^7 (2)[b]	(3.34±1.86)x10^6 (2)	(7.83±5.3)x10^5 (4)	(3.13±2.15)x10^6 (4)
HCV 29T	(3.46±0.29)x10^8 (2)	(1.98±0.22)x10^6 (2)	(6.92±1.32)x10^6 (5)	(2.86±0.56)x10^6 (5)
Hu 549	(1.88±0.46)x10^8 (5)	(0.83±0.26)x10^6 (5)	(9.21±2.45)x10^6 (4)	(2.68±0.72)x10^6 (4)

[a] Binding experiments were analysed using the LIGAND program [29] as modified by McPherson [30].
[b] Number of experiments. Each set of experiments was analysed simultaneously and estimated binding parameters are given as ±SEM [29].

^{125}I-WGA and ^{125}I-sWGA Binding Patterns to TGrII and TGrIII Cellular Proteins

The total, SDS-extracted cellular proteins were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets. The staining of gels with Coomassie blue has shown essentially the same pattern of protein bands for HCV29 and HCV29T cell lines. Some minor differences were observed for Hu549 cells (data not shown). To localize the glycoproteins of total cellular extracts, resolving by SDS-PAGE, 125I-WGA was used. The most intense labeling was observed for glycoproteins with apparent molecular mass ranging from 58-180 kDa (Fig. 3a). The overall staining with lectin was characterized by rather diffuse bands; however, several distinct components were also seen. Among them, the binding of WGA has revealed characteristic differences in the expression of glycoproteins between TGrII cell line - HCV29 and two TGrIII cell lines - HCV29T and Hu549. The TGrIII cell lines showed the presence of two WGA-binding components with apparent molecular mass about 135 and 148 kDa. which were almost totally absent from the TGrII cell line. In contrast, the TGrII cell line expressed an intensive band of apparent molecular mass about 124 kDa. This band was only weakly stained in the remaining cell lines (Fig. 3a). Some differences in the profiles of WGA-binding components were found in cellular extracts obtained from different batches of the same cell line. For example, a rather strong band with apparent molecular mass about 60 kDa was visible only in some glycoprotein extracts from HCV29T and Hu549 cell lines (Fig. 3). Nevertheless, the described differences in the lectin binding profiles among the lines belonging to a particular grade of transformation were found in all analysed samples. When blotted glycoproteins were subjected to mild acid hydrolysis, the binding of WGA was greatly diminished, as compared to untreated glycoproteins (Fig. 3b). Only a few weak bands in the region between 84 and 180 kDa were seen. For cell lines HCV29T and Hu549 two distinct bands with apparent molecular mass 124 and 135 kDa were seen. The 124 kDa band was also visible in the case of HCV29 cells. The profile of binding of 125I-sWGA was essentially the same as was found for the binding of ^{125}I-WGA to desialylated glycoproteins.

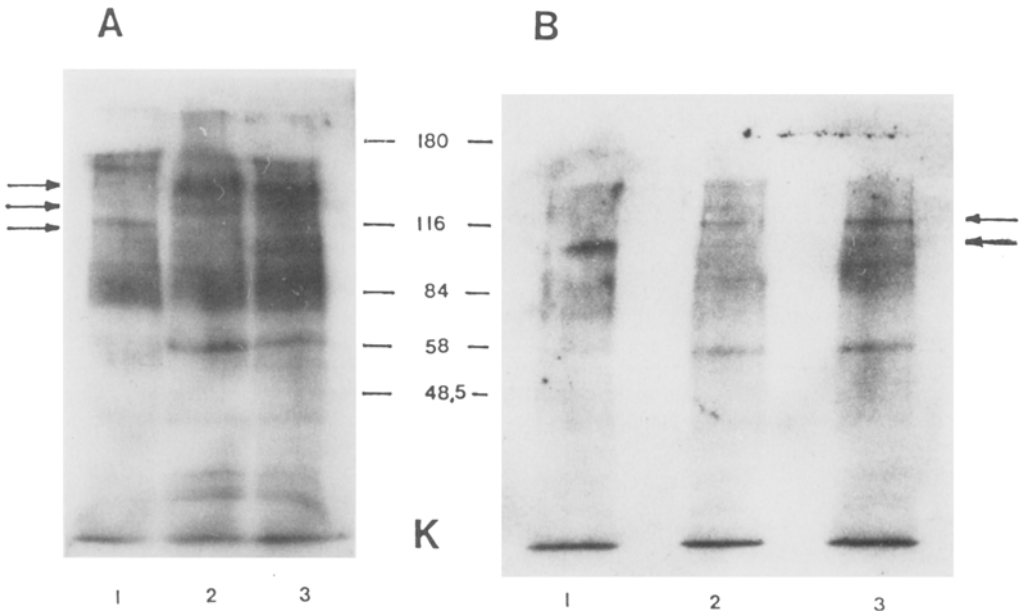


Figure 3. ^{125}I-WGA binding patterns of glycoproteins of human urothelial cell lines. Cell lysates, equivalent to 125 µg cellular protein, were separated by SDS-PAGE under reducing conditions on 7.5% gel and electrophoretically transferred onto nitrocellulose. ^{125}I-WGA-binding components were detected by autoradiography using Kodak X-OMAT R film. Arrows point to the positions of sialoglycoproteins described in the text (see the Results section). (A) = intact glycoproteins; (B) = glycoproteins after mild acid hydrolysis: 1, HCV29 cells; 2, HCV29T cells; 3, Hu549 cells: (K) = molecular standards in kDa.

Discussion

The changes in the expression of surface sialic acid may result from differences in total content or degree of exposure on the cell surface [7, 9, 33]. Moreover, the position/presence of sialic acid may lead to masking or unmasking of different terminal sugar residues (mainly galactose and *N*-acetylgalactosamine [33, 34].

In our previous studies we have shown that tumorigenic and invasive human urothelial cell lines (TGrIII) express more highly branched N-acetyllactosamine type glycans than TGrII cell lines [15]. Similar alterations associated with tumor cell phenotype, connected with an increase in sialic acid content of such oligosaccharide structures were found by Dennis and coworkers. [7], and others [8, 9, 35]. We have also shown, that TGrIII cell lines are more intensively stained by fluorescein-labeled WGA [14]. However, there are also other reports, that the malignant phenotype of human urothelium is characterized by decreased staining with WGA [10]. The results of our studies with the use of ^{125}I-WGA indicate that the

tumorigenic (TGrIII) cell lines HCV29T and Hu549 expressed higher number of WGA binding sites than the non-tumorigenic HCV29 cell line (TGrII) (Table 1). The converse relationship between less and more malignant phenotypes was shown in the apparent association constants, with the highest K_a values for HCV29 cells (Table 1).

WGA binds not only to sialoglycoconjugates but also to glycoconjugates containing *N*-acetyl-D-glucosaminyl residues [36-39]. Therefore, we have also studied the binding of succinylated WGA, which binds solely to *N*-acetylglucosamine residues [21]. The binding of sWGA to tested cell lines did not exceed 2-5% of the unmodified lectin binding, showing that the majority of the receptor sites for WGA is represented by sialic acid residues. The number of sWGA binding sites for HCV29 cells (TGrII) was about ten times lower than for TGrIII cell lines HCV29T and Hu549. This finding is consistent with the differences in the number of binding sites obtained for WGA (Table 1). On the other hand, the differences in K_a among cell lines disappeared, suggesting the more uniform way of binding for sWGA, as compared to WGA binding, to tested cell lines.

The further evidence for the differences in sialic acid-containing glycoconjugates of TGrII and TGrIII cell lines was obtained from ^{125}I-WGA binding to glycoproteins resolved by SDS-PAGE and transferred to nitrocellulose sheets. Distinct quantitative differences were found in sialoglycoproteins with an apparent molecular mass of 124, 135 and 148 kDa in all analysed samples. The components of 124 and 135 kDa were strongly labeled by ^{125}I-WGA in TGrIII cell lines, and were almost totally absent from TGrII cell lines. On the contrary, the strongly labeled WGA-binding protein with an apparent molecular mass of 124 kDa present in TGrII cells was only weakly stained in the TGrIII cell lines. The more intense staining of sialoglycoproteins in the region corresponding to apparent molecular mass 100-200 kDa with WGA was also observed for different malignant cells of rodent origin [11, 12]. After desialylation of glycoproteins by mild acid hydrolysis, two components of apparent molecular mass 135 and 148 kDa for HCV29T and Hu549 cell lines, and one of 124 kDa for HCV29 cells were still visible. A similar profile of binding was observed when untreated glycoproteins were stained with ^{125}I-sWGA. The obtained data suggest that these glycoproteins, besides sialic acid residues, probably possess terminal bisecting N-acetylglucosamine. It has been shown that *N*-glycosidic type glycopeptides without a bisecting *N*-acetylglucosamine residue (*N*-acetylglucosamine linked to the hydroxyl on C4 of the b-mannose) are neither retarded nor bound to WGA [40]. It is in line with our previous findings that TGrIII urothelial cell lines differ from TGrII cell lines by enhanced expression of high molecular mass tri- and tetra-antennary *N*-glycans, which can be represented by bi-antennary glycans with bisecting *N*-acetylglucosamine residues [15].

It is difficult, at this point, to relate directly the observed differences in the sialylation to the biological properties of the studied human urothelial cells with different grades of transformation, when we consider the limited usefulness of *in vitro* model. However, as it was shown by Jacobsen [41], the complement-dependent cytotoxicity against autologous invasive human bladder tumor cells was increased after sialidase treatment. These data imply that increased and/or altered sialylation observed after neoplastic transformation of human urothelial cancer cells can modulate the host immunological response, and indicate, as postulated by Schauer [42], a masking role of the sialic acid residues present on cancer cell surfaces.

Acknowledgements

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