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Research paper

Targeted drug delivery: Binding and uptake of plant lectins using human 5637 bladder cancer cells ☆

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ABSTRACT

In an effort to detect novel strategies in bladder cancer therapy, the potential and the applicability of different plant lectins was investigated using 5637 cells as a model for human urinary carcinoma. The cell–lectin interaction studies were performed with single cells as well as monolayers using flow cytometry and fluorimetry.

As a result, wheat germ agglutinin (WGA) and Ulex europaeus agglutinin (UEA) revealed strongest interaction with single cells demonstrating a high presence of *N*-acetyl-D-glucosamine, sialic acid and α-L-fucose residues on the membrane surface. Considering monolayers, binding of most lectins depended on the culturing period pointing to a change in the glycocalyx composition during cultivation. However, constant binding capacities combined with a high specificity were detected for WGA. Cytoinvasion studies were performed with WGA and revealed a decreased fluorescence intensity at 37 °C as compared to 4 °C, which points to internalisation of the lectin and accumulation in acidic compartments. Intracellular localization was confirmed by addition of monensin that compensates the pH-gradient between acidic compartments and cytoplasm leading to a full reversal of the decline in fluorescence.

According to these findings, some lectins, especially WGA, offer promising features for targeting drugs to bladder cancer cells. This might be interesting for the development of functionalized drug delivery systems for site specific antitumor therapy leading to reduced toxicity, prolonged exposition, and improved efficacy.

1. Introduction

Bladder cancer is one of the most common malignancies, ranking seventh worldwide with higher incidence rates in developed countries [1]. Over the years, advances have been made in diagnosis, therapy, and surveillance resulting in an increasing survival rate of patients but the search for more effective therapies continues [2–4]. Multiple therapeutic approaches especially neoadjuvant chemotherapy are nowadays under investigation [5–7], but the absolute survival benefit is controversially discussed and the clinical application is often limited by severe toxicity. Thus, more targeted delivery of cytotoxic drugs to cancer cells is desirable to circumvent drug toxicity and improve drug efficacy. Among other existing approaches such as transferrin- or folate receptor-targeted drug delivery [8,9], lectin-mediated targeting might be a promising concept [10]. Lectins are known as proteins being capable of binding to certain oligosaccharide moieties [11]. Due to this carbohydrate-specific interaction and alteration of the glycosylation pattern of cells upon malignant transformation [12], lectin-mediated targeting is an encouraging approach towards site specific antitumor therapy. In this context, interactions between different plant lectins and cancer cell lines have been studied in detail [13,14], but in the case of bladder cancer cells there is less knowledge to date. Therefore, the lectin-binding pattern of the human bladder carcinoma cell line 5637 was investigated. This cell line was chosen because of its definition as derived from a grade 2 tumor according to the World Health Organization 1973 classification, nowadays subdivided in low grade and high grade carcinomas, thus representing tumors with a recurrence of 50%, a stage progression of at least 10% and a tumor-related mortality of 5% at the minimum [15].

To assess the interaction between lectins and bladder cancer cells, lectins with distinct carbohydrate specificities were selected: WGA interacts with oligomers of *N*-acetyl-D-glucosamine and sialic acid, whereas peanut agglutinin (PNA) and *Lens culinaris* agglutinin (LCA) bind to galactosamine and α-mannose, respectively. *Solanum tuberosum* lectin (STL) also recognises *N*-acetyl-D-glucosamine, *Dolichus biflorus* agglutinin (DBA) interacts with *N*-acetyl-D-galactosamine-residues, and finally UEA binds to α-L-fucose structures.

The aim of this work was to evaluate the binding rate and specificity of the selected lectins. This estimation of the glycosylation

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pattern of human bladder cancer cells should provide the basis for the development of lectin-grafted drug delivery systems. Additionally, as most advantageous for drug delivery, the underlying mechanism of a possible cellular uptake of a certain lectin will be elucidated.

2. Materials and methods

2.1. Chemicals

Fluorescein-labelled lectins from Triticum vulgare (molar ratio fluorescein/protein (F/P) = 5.4), *Arachis hypogaea* (F/P = 4.7), *Dolichus biflorus* (F/P = 6.3), *Lens culinaris* (F/P = 6.3), *Solanum tuberosum* (F/P = 2.9) and *Ulex europaeus* (isoagglutinin I, F/P = 4.0) were purchased from Vector laboratories (Burlingame, USA).

All other chemicals were obtained from Sigma (St. Louis, USA), unless otherwise specified.

2.2. Cell culture

The human urinary bladder carcinoma cell line 5637 was purchased from the American Type Culture Collection (Rockeville, USA). Cells at passages 47–55 were cultured in RPMI-1640 medium containing 10% fetal calf serum (Biochrom AG, Germany), 4 mM L-glutamine, and 150 μg/ml gentamycin in a humidified 5% CO_2/95% air atmosphere at 37 °C and subcultured by trypsination.

For monolayer studies, cells were seeded on TC-treated 96-well microplates (Greiner, Austria) at a density of 1.7×10^4/well. Culture medium was changed every other day and monolayers were used 4–11 d after seeding.

2.3. Flow cytometry

Flow cytometric measurements were carried out on an Epics XL-MLC analytical flow cytometer (Coulter, USA). Cell-bound fluorescence intensities were analysed using a forward versus side scatter gate to detect the single cell population. For each measurement 3000 cells were accumulated. Fluorescence emission was detected at 525 nm (10 nm bandwidth) after excitation at 488 nm. For further calculations, the mean channel number of the logarithmic fluorescence intensities of individual peaks was used.

2.4. Determination of the lectin-binding capacity of bladder carcinoma cells 5637

Binding of lectins to the surface of 5637 cells was investigated using both single cells and monolayers.

As to single cell experiments, 5637 cells were harvested by trypsination, collected by centrifugation, and processed immediately. Fifty microliters of cell suspension (3×10^5 cells) were mixed with 50 μl of the respective lectin in PBS (1.56–100 pmol lectin, serial dilutions). After incubation for 5 min at 4 °C, cells were washed with PBS to remove unbound lectin. Then, the relative cell-associated fluorescence intensity (RFI) was determined by flow cytometry. In order to estimate the autofluorescence of the cells, unlabelled cells were included and the obtained data subtracted from the binding data quoted.

As to monolayers, cell layers were used 4, 6, 8 or 11 d after seeding. They were washed prior to incubation with 50 μl lectin solution (1.56–50 pmol, serial dilutions) for 10 min at 4 °C. Unbound lectin was removed and the RFI was determined using a fluorescence microplate reader (Spectrafluor Fluorometer, Tecan, Austria). The autofluorescence of the plate and cells was considered by reading the RFI of cell layers incubated for 10 min at 4 °C with PBS at 485/525 nm.

Table 1
Specificity of the lectins used in the competitive binding assays

Lectin	MW	Carbohydrate specificity	Inhibitory sugar
WGA	36,000	GlcNAc, NANA	*N*-acetyl-D-glucosamine, Chitotriose
STL	100,000	GlcNAc	*N*-acetyl-D-glucosamine, Chitotriose
LCA	49,000	α-Man, α-Glc, α-GlcNAc	D-mannose
UEA	63,000	α-L-Fuc	L-fucose
DBA	120,000	α-D-GalNAc, Gal	*N*-acetyl-D-galactosamine
PNA	110,000	β-D-Gal-D-GalNAc, β-D-GalNAc, Gal	D-galactosamine

GlcNac, *N*-acteyl-D-glucosamine; NANA, *N*-acetyl-neuraminic acid; α-Man, α-mannose; α-Glc, α-glucose; α-L-Fuc, α-L-fucose; α-D-GalNAc, *N*-acetyl-α-galactosamine; Gal, galactose.

Specificity of lectin binding was verified by competitive inhibition with the complementary carbohydrates (Table 1). Fifty microliters of cell suspension (3.0×10^5 cells), 100 μl of a dilution series of the inhibitory sugar (3.1–100 μg, for LCA 3.1–1600 μg, serial dilutions) and 50 μl of the lectin solution (40 μg/ml) were mixed, incubated for 1 h at 4 °C and processed as described above. Monolayers, cultivated for 7 days, were incubated with 100 μl of the lectin-combining carbohydrate (3.1–100 μg, serial dilutions) and 50 μl of the lectin (40 μg/ml) for 10 min at 4 °C.

2.5. Internalisation of cell surface-bound WGA

Fifty microliters of cell suspension (3.0×10^5 cells) were incubated with 50 μl WGA solution (10 μg/ml PBS) for 5 min at 4 °C. Unbound lectin was removed and the cell suspension was further incubated for 0–240 min at 37 °C and 4 °C, respectively. After determining the RFI by flow cytometry, 40 μl of a 2.4 mM monensin solution were added, followed by incubation at RT for 3 min. Finally, the RFI was determined again. Upon the treatment of cells with monensin, the pH gradient between acidic compartments and the cytosol is compensated, abolishing the quench of fluorescein-labelled WGA provided its accumulation within acidic compartments.

3. Results

3.1. Lectin-association with 5637 cells

For comparison of the data obtained, RFI values of each lectin were related to an apparent conjugation number of 1 mol fluorescein per mole lectin. At the single cell level, the resulting binding curves of each lectin revealed an increase of cell-bound fluorescence intensity with an increase in the amount of lectin added (Fig. 1). The binding capacity of 5637 cells was most pronounced

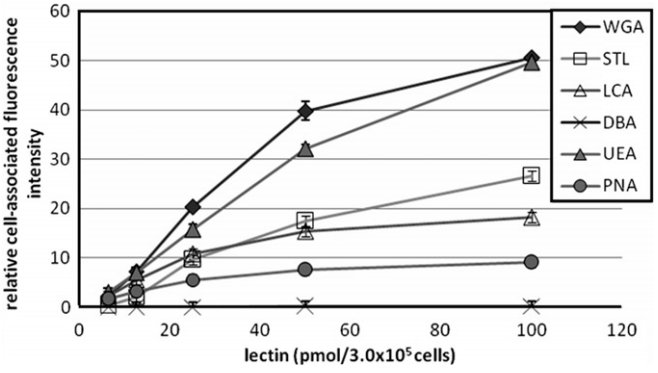

Fig. 1. Lectin binding to 5637 single cell at 4 °C. The fluorescein-labelled lectins associated with the cell surface were related to an apparent F/P ratio of 1 (mean ± SD, $n = 3$).

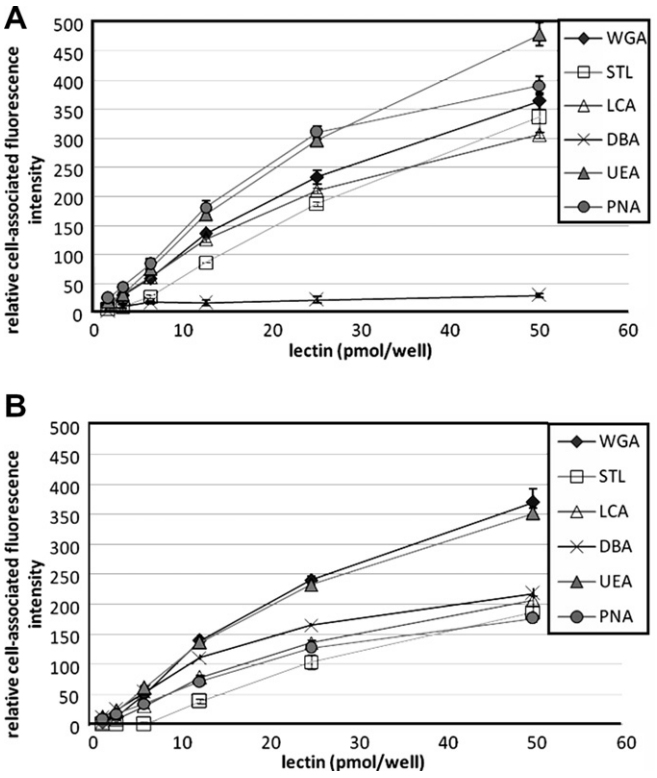

Fig. 2. Lectin binding to 5637 cell monolayers cultivated for 4 d (A) or 11 d (B) at 4 °C. The fluorescein-labelled lectins associated with the cell surface were related to an apparent F/P ratio of 1 (mean ± SD, $n = 4$).

in the case of WGA and UEA amounting to 2.2 ± 0.1–50.5 ± 0.3 RFI (WGA) and 3.0 ± 0.7–49.7 ± 0.1 RFI (UEA).

Considering the monolayers, the amount of cell-bound lectins changed with the cultivation time. For monolayers grown for 4 days, a pronounced association was observed for UEA and PNA with fluorescence intensities between 19.3 ± 2.0–479.0 ± 20.0 RFI (UEA) and 24.4 ± 2.3–390.8 ± 16.2 RFI (PNA, Fig. 2A), whereas after 11 days WGA-binding prevailed at concentrations between 12.5 and 50 pmol as indicated by 140.0 ± 3.1–371.2 ± 22.0 RFI (Fig. 2B). For UEA, PNA, STL and LCA, the surface-bound amount of lectin decreases with cultivation time. WGA and DBA, however, featured a different cultivation time-dependent RFI profile. At a concentration of 50 pmol, for WGA constant binding capacities were detected with intensities ranging from 364.1 ± 14.2 to 371.2 ± 22.0. The interaction between DBA and 5637 cells increased notedly between days 4 and 11 in culture as indicated by the determined fluorescence values of 29.4 ± 2.9 at day 4 and 218.7 ± 9.0 at day 11.

Specificity of the lectin–cell interaction was investigated by competitive inhibition of the lectin-binding to 5637 cells. Depending on the amount of carbohydrate added to the cells, the carbohydrate binding domains of the lectins are blocked and thus inhibited from binding to the cell membrane. The amount of cell-bound lectin decreased in the presence of 3.1–100 μg lectin-specific carbohydrate, as compared to the control which indicates for specificity (Fig. 3). The flattening of the inhibition curves at 89.7% in the case of WGA, 83.9% for STL, and 78.3% in the case of PNA pointed to a high degree of specific binding. For UEA and DBA, the contribution of non-specific binding was notedly higher as indicated by inhibition curves levelling off at concentrations between 62.6% and 55.8%. For LCA, however, it was necessary to increase the concentration of the carbohydrate to 1600 μg to approach the inhibition rates of about 50%.

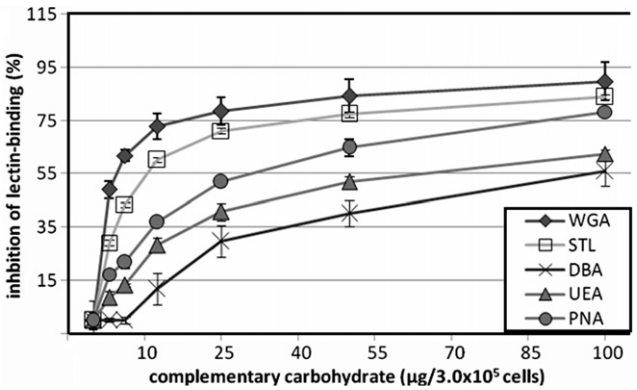

Fig. 3. Competitive inhibition of lectin binding to 5637 single cells by addition of increasing amounts of complementary carbohydrate (mean ± SD, $n = 3$).

Monolayers cultivated for 7 days revealed similar inhibition profiles (data not shown).

3.2. Internalisation of cell-bound WGA

During the pulse incubation at 4 °C for 5 min, WGA was allowed to interact with the cell membrane followed by the removal of unbound lectin to guarantee identical starting conditions. When cells were chase-incubated at 37 °C, a time-dependent decrease in RFI was observed finally yielding a difference of 43.3 ± 0.6 RFI (Fig. 4) as compared to the initial values. Upon the addition of monensin, these reduced fluorescence intensities were restored to approach those of the control sample measured immediately after the pulse-incubation with WGA (0 min, 69.3 ± 2.7 RFI).

When the same assay was performed at 4 °C, no differences between the RFI prior and after monensin addition were detectable throughout the incubation time.

4. Discussion

In an effort to develop novel strategies for bladder cancer therapy, at first the interaction of different plant lectins with the cell membrane of 5637 cells was elucidated. As depicted from saturation studies, the lectin-binding to single cells increased as follows: DBA < PNA < LCA < STL < UEA < WGA (Fig. 1). Accordingly, the carbohydrate pattern is reflected by high abundance of *N*-acetyl-D-glucosamine and sialic acid. These complementary carbohydrates of WGA seem to prevail and to be highly accessible, followed by α-L-fucose > mannose > galactosamine and *N*-acetyl-D-galactos-

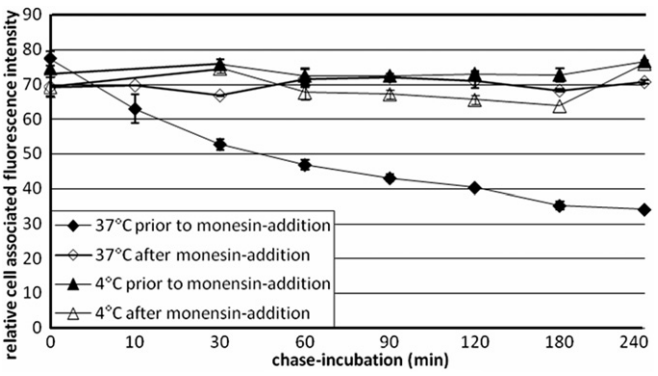

Fig. 4. Relative cell-associated fluorescence intensities of WGA-loaded 5637 single cells prior and after monensin addition during incubation at both 4 °C and 37 °C by time up to 4 h (mean ± SD, $n = 3$).

amine. In the case of tissue formation by growing monolayers, the glycosylation pattern changes by cultivation time except for WGA. The extent of interaction mostly declines slightly over prolonged cultivation as detected by a decrease of the RFI. After 4 days, UEA and PNA showed the highest binding capacities, whereas after 11 days WGA interacted best (Fig. 2A and B). Interestingly, DBA had a 7.4-fold higher binding capacity after 11 days in comparison to cell layers cultivated for 4 days, indicating that the amount of *N*-acetyl-D-galactosamine structures increased upon prolonged culture. In contrast, *N*-acetyl-D-glucosamine residues accessible for STL showed to be down regulated. As the binding capacity of WGA, which also recognises *N*-acetyl-D-glucosamine structures and moreover sialic acid, rested stable during cultivation, an increased expression of sialic acid-containing oligosaccharides is supposed.

Besides high binding rates, binding specificity would be advantageous for carbohydrate-mediated targeting. WGA exhibited highest binding specificity yielding 89.7% inhibition (single cells) and 95.6% (monolayer) of glycosylated membrane proteins containing the *N*, *N*′, *N*″-triacetylchitotriose-motif. For the other lectins, the specificity at the single cell level decreased in the following manner STL > PNA > UEA > DBA > LCA. In case of the monolayers, it declines in the order PNA > STL > DBA > UEA > LCA. Therefore UEA, which had shown a good binding capacity, was not further taken into account due to the high amount of unspecific binding.

As WGA displayed a high binding capacity together with a high specificity (Figs. 1–3), this lectin was chosen to study cytoinvasion as beneficial for targeted drug delivery. Upon chase-incubation at 4 °C, the RFI remained constant indicating irreversible binding of WGA to the cell surface without any evidence for the detachment of membrane-bound lectin. At 37 °C, however, fluorescence intensities continuously decreased pointing to internalisation of cell-bound lectin and enrichment within acidic compartments since the quantum yield of fluorescein is quenched in acidic environment. To verify this assumption, assays in the presence of monensin were performed which compensates the pH-gradient between acidic compartments and cytoplasma. After the addition of monensin, the pH-dependent quench of the acid-sensitive label was fully restored (Fig. 4). Again, no fluorescent WGA was detectable in the supernatant allowing to calculate the fraction of WGA entering the lysosomal pathway [14]. As the fluorescence emission of fluorescein is reduced to about 10% at lysosomal pH, the results indicate that after 10 min 10% and after 60 min already 40% are located within acidic compartments of the cell. At the end of the incubation period, about 60% of the cell-associated WGA are accumulated in the lysosomes. Additionally, the avidin-biotin concept [16] was applied to reveal the amount of internalised lectin: at the single cell level 50% were taken up within 10 min, whereas for monolayer assays 50% were internalised after 4 h (data not shown). All in all, cytoinvasion studies of WGA using single cells reveal that after 10 min about 25% of the internalised lectin are accumulated in acidic compartments, whereas after 4 h already 67% of the entered lectin are located there.

Since uptake of WGA can not be mediated by binding to immobile *N*-acetyl-D-glucosamine-containing oligosaccharides present at the surface of 5637 cells, specific carbohydrate-containing receptors must be involved in the uptake-process. Two concepts are conceivable: involvement of the epidermal growth factor receptor (EGF-R) and/or the CD44 receptor.

EGF-R expression was found in the 5637 cell line by Van der Poel et al. [17]. Vale and Shooter [18] observed that the binding of I125-EGF decreased to less than 50% after preincubation of PC12 cells with either WGA or concanavalin A, and Lochner et al. showed the saturable binding of WGA to isolated receptors and biomimetic membranes prepared from Caco-2 and A-431 cells [19]. According to these findings, the EGF-R might also be involved in the binding and transport of WGA in bladder cancer cells as it is known to be overexpressed in a high number of tumors including the bladder.

Another possible route includes the CD44 receptor. Multiple CD44 variants were found in the 5637 cell line [20]. CD44 is known to act as a receptor for hyaluronic acid and as WGA specifically binds to *N*-acetyl-D-glucosamine, which is typically found in hyaluronic acid, its transport might also be mediated by this receptor.

As to the toxicity of WGA, which is controversially discussed, Gabor et al. [21] came to the conclusion that the amounts of lectins as necessary for glycotargeting of prodrugs or colloidal carrier systems are in the microgram range so that toxic effects should not be provoked.

Since prolonged and targeted adhesion of a drug delivery system is a key for improved local action of cytostatics reducing the damage of non-diseased tissue, the cytoadhesive effects of WGA are expected to mediate prolonged and local action especially counteracting the loss of drug by urinary excretion. However, the presence of sugars in the urine may have an impact on the efficiency of the lectin binding which should be considered in further investigation. But for the intended use as a bladder irrigation/instillation, this presence should be of only moderate impact. The cytoinvasive effects are supposed to mediate the uptake of conjugated drugs or drug-loaded colloids which can further enhance the therapeutic effect of the cytostatic agent.

To enforce the feasibility of the lectin-mediated targeting concept, a comparison to non-malignant human urothelial cells is inevitable. To date, there are promising hints in the literature, that the expression of glycoconjugate structures in normal and cancerous human urothelium is quite different resulting in increased numbers of lectin- binding sites upon malignant transformation of the urothelium [22,23]. Ongoing from successful ex-vivo studies, animal experiments should be performed e.g. according to ith Wistar rats a former experimental setup, where the rats were treated with *N*-butyl-N(4-hydroxybutyl)nitrosamine (BBN) in order to induce bladder cancer and various lectins including WGA showed a specific affinity to BBN-induced carcinoma [22]. All in all, following on from this work WGA-grafted drug-delivery systems might open an interesting approach towards improved bladder cancer therapy.

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