ELSEVIER

International Journal of Pharmaceutics 221 (2001) 35–47

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

The Interaction between wheat germ agglutinin and other plant lectins with prostate cancer cells Du-145

Franz Gabor, Ursula Klausegger, Michael Wirth *

Institute of Pharmaceutical Technology and Biopharmaceutics, The University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria

Received 18 December 2000; received in revised form 5 March 2001; accepted 12 March 2001

Abstract

The bioadhesive properties of fluorescein-labeled plant lectins with different carbohydrate specificities were investigated by flow cytometry at 4 and 37°C using Du-145 prostate cancer cells. At both temperatures the lectin association rate increased following the order: *Dolichos biflorus* agglutinin (DBA) < peanut agglutinin < *Ulex europaeus* isoagglutinin I < *Lens culinaris* agglutinin < *Solanum tuberosum* lectin ≪ wheat germ agglutinin (WGA), reflecting the glycosylation pattern of Du-145 cells. Both, the BSA-binding capacity of the cells referring to nonspecific binding and inhibition studies using the complementary carbohydrate, assured specificity of the lectin–cell interactions except for DBA. The WGA-association rate of Du-145 cells was dependent on temperature indicative for cellular uptake of membrane-bound WGA. Intracellular enrichment of WGA was confirmed by confocal microscopy. As resulted from experiments in presence of ouabain active transport mechanisms were involved in cellular uptake of WGA. Equilibration of the intracellular pH with monensin pointed to accumulation of intracellular located WGA within acidic compartments of Du-145 cells such as the lysosomes or the trans-Golgi complex. Consequently the interaction of WGA with Du-145 cells at 37°C is a one way process due to immediate active transport of membrane-bound lectin into acidic compartments of prostate cancer cells.

Keywords: Du-145; Lectin; Wheat germ agglutinin; Bioadhesion; Flow cytometry; Active transport

1. Introduction

When Robert Stillmark observed in 1888 agglutination of red blood cells by castor bean extracts, he opened a wide area of applications of lectins. Over the intervening years plant lectins, recently defined as proteins possessing at least one non-catalytic domain, which binds reversibly to specific mono- or oligo-saccharides (Peumans and Van Damme, 1995), found broad application in cytochemistry especially in hematology and histochemistry (Danguy et al., 1998). Beyond this, lectins recently gained some attention as the second generation of bioadhesives in drug delivery (Lehr, 2000). Whereas mucoadhesive polymers are already used as excipients in pharmaceutical for-

* Corresponding author. Tel.: +43-1-427755406; fax: +43-1-42779554.

E-mail address: michael.wirth@univie.ac.at (M. Wirth).

0378-5173/01/$ - see front matter
PII: S0378-5173(01)00650-0

mulations, a lot of questions remains to be answered in case of cytoadhesive lectins such as the elucidation of mechanisms involved in lectin–cell interaction. Especially in view of detection of about 3% of tomato–lectin coated nanoparticles in systemic circulation after peroral administration in rats (Florence et al., 1995), lectin-mediated cytoadhesion can be followed by transcytosis due to the lectin-coat, which increases systemic availability of the delivery system. Whereas cellular uptake of lectins was confirmed by confocal laser scanning microscopy (Wirth et al., 1998a), little is known about transport mechanisms and cellular fate of lectins considering lectins as pharmaceutical excipients. The lectin-binding characteristics of the intestinal cell lines Caco-2, HT-29, HCT-8 and human colonocytes were studied in detail, but there is less knowledge about the interaction between lectins and epithelial prostate cancer cells to date (Jepson et al., 1995; Gabor et al., 1997, 1998). As every third malignant tumor of 50–70-year-old men in the western world is prostate cancer, Du-145 cells were used to get an idea of the applicability of the lectin-approach in the field of site specific antitumor therapy.

The Du-145 cell line originates from human prostate adenocarcinoma metastatic to the brain (Stone et al., 1978). The adherent growing epithelial cells reveal microvilli, tonofilaments and desmosomes forming loose monolayers. Electron microscopy of the original tumor tissue and the tissue culture cell line showed remarkable similarity in cell organelle structure and only minor quantitative differences, in that the cultured cells had less rough endoplasmic reticulum, less junctional complexes and fewer secretory granules (Mickey et al., 1977). Du-145 cells appear to be neither hormone-sensitive nor-dependent, although there are few reports of hormone response in vitro.

For investigation of the lectin–prostate cancer cell interaction by flow cytometry, a panel of lectins with different carbohydrate specificity was selected: wheat germ agglutinin (WGA) from *Triticum vulgare* interacts with oligomers of *N*-acetyl-D-glucosamine and sialic acid, whereas succinoylated wheat germ agglutinin (WGAs) and *Solanum tuberosum* lectin (STL) from potato tubers binds to *N*-acetyl-D-glucosamine exclusively. The lectin from horse gram (*Dolichos biflorus* agglutinin, DBA) recognizes *N*-acetyl-galactosamine-residues, whereas the lectin from furze seeds (*Ulex europaeus* isoagglutinin I, UEA-I) binds to α-L-fucose-containing oligosaccharides. Additionally the peanut agglutinin (PNA) from *Arachis hypogea* and the lentil lectin from *Lens culinaris* (LCA) interacting with galactosamine- and α-mannose-residues were included.

The purpose of this paper is to outline the binding characteristics and binding specificity of several selected lectins with different carbohydrate specificity. Additionally focus is set on mechanisms involved in uptake and cellular fate of WGA.

2. Materials and methods

2.1. Chemicals

The fluorescein labeled lectins from *Triticum vulgare* (molar ratio fluorescein/protein (F/P) = 3.2, *S. tuberosum* (F/P = 2.9), *D. biflorus* (F/P = 5.2), *A. hypogea* (F/P = 4.7), *U. europaeus* (UEA-I, F/P = 4.0) and *L. culinaris* (F/P = 6.3) were purchased from Vector laboratories (Burlingame, USA) and contained > 98% active conjugate and no free fluorescein. Tissue culture reagents were from Sigma (St. Louis, MO) and Gibco (Vienna, Austria).

N,*N*′,*N*″-triacetylchitotriose, *N*-acetyl-D-galactosamine, D-galactosamine, α-L-fucose, D-mannose and fluorescein labeled bovine serum albumin (BSA, F/P = 12) were from Sigma; all other chemicals were of analytical grade and purchased from Merck (Darmstadt, G). Isotone phosphate buffered saline (PBS) was used throughout the experiments.

2.2. Tissue culture

The human prostate adenocarcinoma cell line Du-145 was obtained from the American Type Culture Collection (Rockville, ML, USA). Cells were grown in culture medium consisting of RPMI-1640 with 10% fetal calf serum (FCS), 4

mM L-glutamine and 150 μg/ml gentamycine in a humidified 5% CO_2/95% air atmosphere at 37°C and subcultured by trypsination.

2.3. Determination of the lectin-association with prostate carcinoma cells

Using a 96-well microplate, 100 μl PBS and 50 μl of a dilution series of labeled lectins in PBS (4, 2, 1, 0.5 μg per well) were mixed followed by addition of 50 μl cell-suspension (3.0×10^5 cells) in PBS. The cells were resuspended and incubated for 1 or 3 h at 4 or 37°C, respectively. Cells were collected by centrifugation (1000 rpm, 5 min) and 130 μl of the supernatant was discarded. After addition of 130 μl PBS, washing was repeated in the same manner. Cells were resuspended in 1 ml Cell Pack and assayed by flow cytometry. Negative controls were included in every experiment consisting of unlabeled cells for estimation of autofluorescence. Each concentration was tested in triplicate and the dilution experiments were repeated at least twice in each case.

As a control for estimation of non-specific binding aliquots were prepared as above but using a dilution series of fluorescein labeled BSA (F/P = 12) instead of the lectins.

For comparison of the data obtained, the mean relative fluorescence intensity of each individual lectin is related to an apparent conjugation number of 1 mol fluorescein per mol lectin.

2.4. Carbohydrate-binding specificity of the lectins

To assess the carbohydrate-specificity of lectin-binding to Du-145 cells, 100 μl of a dilution series of the lectin-specific carbohydrate (100–3.1 μg, serial dilution), 50 μl of cell-suspension (3.0×10^5 cells) in PBS and 50 μl PBS containing 2 μg lectin were mixed. After incubation for 1 h at 4°C, the samples were processed as described above.

2.5. Effect of ouabain on Du-145 associated F-WGA

As found out in preliminary experiments 10 μM ouabain were most effective to inhibit active intracellular uptake of WGA without affecting viability of the cells. The inhibitory effect of ouabain lasted up to 45 min. Accordingly, a mixture of 50 μl cell suspension (3.0×10^5 cells in PBS), 20 μl 100 μM ouabain and 80 μl PBS were preincubated for 15 min at 37°C. After addition of 50 μl of PBS containing 2 μg F-WGA, the cells were resuspended and incubated for 30 min at 37°C. Cells were washed to remove unbound lectin as described above and analyzed by flow cytometry either immediately after washing or after storage for 30 min at 37°C.

The same assay as above but replacing the ouabain-solution by PBS served as a control.

2.6. Influence of monensin on fluorescence intensity of cell-associated F-WGA

In order to estimate the influence of the carboxylic ionophore monensin on fluorescence intensity of cell-associated F-WGA, 50 μl cell-suspension (3.0×10^5 cells in PBS) and a mixture of 50 μl of PBS containing 2 μg F-WGA and 100 μl PBS were resuspended and preincubated for 30 min at 37°C. The cells were washed twice and stored at 37°C. At regular intervals 10 μl of 2.4 mM monensin in ethanol were added followed by flow cytometric analysis after 7 min of incubation.

Results were compared with an assay as above but lacking addition of monensin.

2.7. Flow cytometry

Flow cytometric measurements were carried out using an Epics XL-MLC analytical flow cytometer (Coulter, FL). Cell-bound fluorescence intensity of the single-cell suspension was determined using a forward versus side scatter gate for the inclusion of single cell populations and exclusion of debris and cell aggregates. Fluorescence was detected at 525 nm (10nm bandwidth) and the mean channel number of the logarithmic fluorescence intensities of individual peaks was used for further calculations. Amplification of the fluorescence signals was adjusted to put the autofluorescence signal of unlabeled cells in the first decade of the 4-decade log range resulting in gain 650 for each experiment. For each measurement 3000 cells were accumulated.

2.8. Confocal microscopy

Cells were stained by incubation of 150 μl cell-suspension (1.0×10^6/ml PBS) with 50 μl solution of F-WGA (80 μg/ml PBS) for 1 h at 4 and 37°C, respectively. Cells were spun down (5 min, 4°C, 1000 rpm), washed twice as described above and mounted for microscopy. Confocal images of fluorescent labeled cells were obtained using a Zeiss Axiovert confocal microscope. Transmission light and fluorescence pictures were acquired at $60\times$ magnification and the black level (background offset) of the green fluorescence detector was adjusted to eliminate any autofluorescence of unstained cells.

3. Results

3.1. Viability of prostate cancer cells

Loss of viability causes alterations of extension as well as surface shape of cells. As flow cytometry detects forward and side scattering of cells, upon loss of viability a new population representing non-viable cells appears in the histogram. The Du-145 cells were found to be viable for 24 h at room temperature, but by time viability decreased from 82 (2 days storage) to 47% (3 days storage) and 35% (4 days storage), respectively.

3.2. Lectin-association with Du-145 cancer cells

To estimate the lectin-binding capacity of prostate cancer cells, increasing amounts of fluorescein-labeled lectins were allowed to interact with a fixed number of Du-145 cells at 4 and 37°C, respectively.

Independent from carbohydrate specificity of the lectin, at 4°C the cell-bound fluorescence intensity increased with addition of increasing amounts of lectin. Incubating Du-145 cells with increasing amounts of WGA resulted in increasing cell-associated fluorescence intensity ranging from 108 ± 12 to 395 ± 16 (mean $\pm$ S.D., $n = 3$) as compared with autofluorescence of Du-145 cells yielding 0.1 ± 0.01 ($n = 12$). But the amount of Du-145 bound lectin was strongly dependent on sugar-specificity of each individual lectin. When 1 μg lectin was applied to a fixed number of cells at 4°C and the mean cell-bound fluorescence intensity was related to an apparent conjugation number of 1 mol fluorescein per mol protein, the lectin-binding capacity of the Du-145-membrane was 25-fold higher in case of WGA, eightfold higher in case of STL, 2.5-fold higher in case of

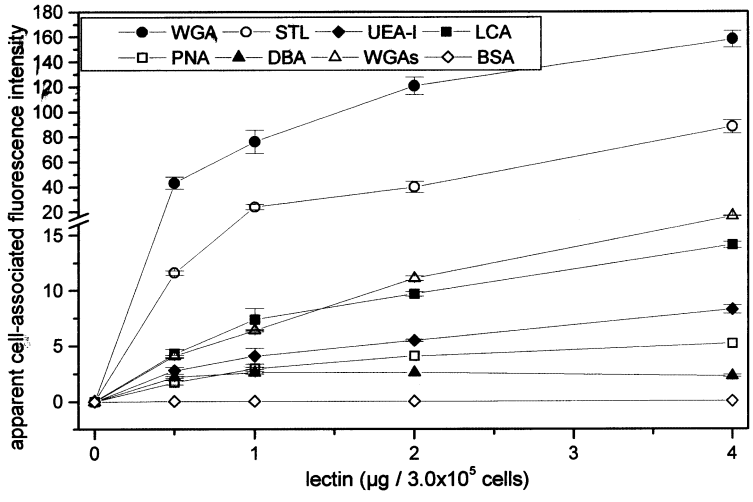

Fig. 1. Saturation analysis of lectin-binding sites on Du-145 cells with fluorescein-labeled lectins at 4°C in comparison to BSA related to an apparent F/P-ratio = 1 (mean $\pm$ S.D., $n = 6$).

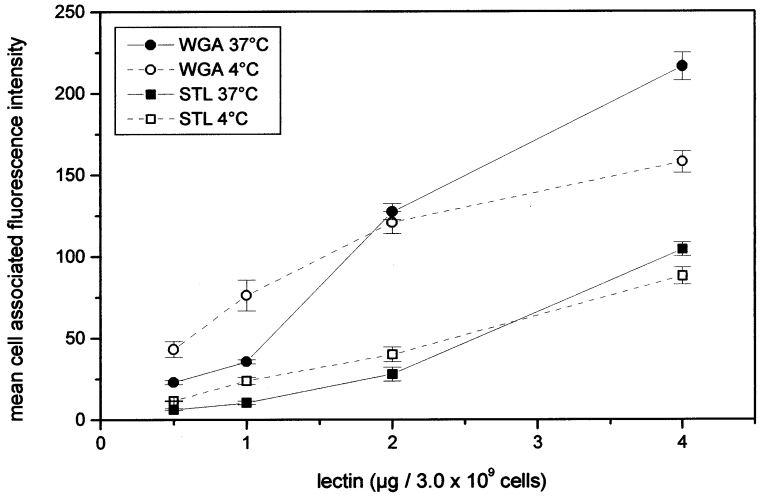

Fig. 2. Association of fluorescein-labeled wheat germ agglutinin (WGA) and *Solanum tuberosum-lectin* (STL) with Du-145 cells at 4 and 37°C, respectively (mean ± S.D., $n = 3$).

LCA, 2.1-fold higher in case of WGAs and 1.4-fold higher in case of UEA-I than that of PNA and DBA, respectively (Fig. 1). The histograms revealed homogenous staining of all prostate cancer cells and formation of unstained subpopulations was not observed.

Whereas lectin-binding to the membrane is determined predominantly at 4°C, at 37°C lectin-binding to the glycocalyx as well as internalization of the membrane-bound lectin can occur. Similar to membrane-binding at 4°C, the lectin-association rate at 37°C increased concurrent with increasing the lectin-concentration. Accordingly, lectin-association followed the order: WGA ≫ STL > LCA > UEA-I > PNA > DBA. But in case of UEA-l, LCA, PNA, DBA and at low concentrations of STL as well as WGA the relative cell-associated fluorescence intensity was lower than that at 4°C, whereas at high concentrations of WGA and STL intensified staining was observed (Fig. 2, Table 1).

3.3. Specificity of binding

To exclude the influence of temperature on lectin association, the specificity of the lectin–cell interaction was investigated at 4°C. Assuming that the BSA-binding rate of prostate cancer cells represents the extent of nonspecific interaction, an utmost extent of 0.05% (WGA), 0.16% (STL), 0.97% (UEA-I), 0.54% (LCA), 1.33% (PNA) and 1.54% (DBA) of membrane-binding might be attributed to nonspecific interaction (see Fig. 1).

Specificity of the lectin–cell interaction was investigated by competitive inhibition of lectin-binding to the prostate cancer cells. Dependent on the amount of complementary carbohydrate added, the carbohydrate-combining site of the lectin is blocked partially and inhibited from binding to the cell-membrane. Except for DBA the amount of cell-bound lectin decreased upon addition of increasing amounts of the corresponding carbohydrate indicating for specificity of the lectin–membrane interaction (Fig. 3). For comparison of the data obtained, the amount of carbohydrate necessary for 50% inhibition of lectin-binding (IC_{50}) was calculated from the plots (Table 2). The rather high amounts of the corresponding monosaccharides as necessary to inhibit lectin-binding to the cells point to high specificity of the lectin–cell interaction. The affinity of LCA to Du-145 was found to be about four-fold higher than that of UEA-I or about 20 times higher than the PNA–prostate cancer interaction. But this must not be compared with Du-145 affinity of WGA and STL, since the inhibitory potency of

the trisaccharide is about 3700-fold higher than that of the monosaccharide (Liener et al., 1986). As inhibition of binding by considerable high amounts of *N*-acetyl-D-glucosamine was rather low, *N*,*N*′,*N*″-triacetylchitotriose was used in this assay. According to Table 2 the affinity of STL to Du-145 cells is about three times higher compared with WGA.

Since the affinity as well as the binding rate of WGA pointed to high cytoadhesiveness of this lectin, the WGA–Du-145 interaction was elucidated in more detail.

3.4. Effect of temperature and lectin-concentration on the WGA–Du-145 interaction

As already observed in the saturation assay, there was a contradictory influence of incubation temperature on cell-associated fluorescence intensity after incubation of Du-145 cells with F-WGA. When the binding-assay was performed as described above with a fixed number of cells at 4 or 37°C, but varying the incubation period and lectin-concentration, the cell-associated fluorescence intensity was found to be dependent on F-WGA-concentration and duration of exposition to the cells. At 37°C and high F-WGA concentrations (4 μg F-WGA per well) the cell-associated fluorescence increased within 1 h to 137% as compared with 4°C (Fig. 4). At medium F-WGA concentrations (2 μg F-WGA per well) there was no significant difference in cell-associated fluorescence intensity between the two levels of temperature. Finally at low amounts of the fluorescein-labeled lectin, the cell-associated fluorescence intensity evenly decreased within 1 h to 54% (0.5 μg F-WGA per well) or 46% (1 μg F-WGA per well) as compared with 4°C.

Since the decrease in cell-associated fluorescence intensity at 37°C might be due to detachment of initially cell-bound lectin, Du-145 cells

Table 1
Cell-associated mean relative fluorescence intensities (mean ± S.D., $n = 3$) after incubation of 3.0×10^5 Du-145 cells with fluorescent labeled WGA, STL, LCA or UEA-I for 1 or 3 h at 4 and 37°C, respectively

μg lectin/3.0×10^5 cells	Incubation for 1 h		Incubation for 3 h	
	At 4°C	At 37°C	At 4°C	At 37°C
F-WGA				
0.5	108.0 ± 12.0	57.4 ± 3.2	137.5 ± 2.5	76.2 ± 1.7
1.0	190.5 ± 23.5	89.0 ± 3.0	226.0 ± 8.4	121.4 ± 1.6
2.0	302.0 ± 17.0	318.5 ± 12.3	323.3 ± 2.1	291.0 ± 3.1
4.0	394.5 ± 16.5	540.6 ± 21.4	428.1 ± 2.8	538.7 ± 6.8
F-STL				
0.5	33.5 ± 0.5	18.3 ± 1.7	31.4 ± 0.4	17.3 ± 0.3
1.0	69.5 ± 6.5	30.4 ± 2.8	46.6 ± 1.3	24.5 ± 0.6
2.0	116.0 ± 13.0	80.8 ± 12.4	75.2 ± 2.9	45.5 ± 1.1
4.0	255.0 ± 15.0	302.6 ± 12.4	159.3 ± 4.0	146.5 ± 4.6
F-LCA				
0.5	27.0 ± 2.2	16.6 ± 1.4	31.6 ± 0.4	26.2 ± 0.2
1.0	46.6 ± 6.6	27.3 ± 0.5	48.0 ± 0.3	36.1 ± 0.3
2.0	61.4 ± 1.6	44.5 ± 3.6	65.5 ± 0.9	52.9 ± 0.4
4.0	88.6 ± 1.8	54.8 ± 0.4	81.2 ± 0.4	69.7 ± 0.5
F-UEA-I				
0.5	11.0 ± 1.0	89 ± 0.7	71.0 ± 2.8	36.4 ± 0.4
1.0	16.5 ± 2.8	11.7 ± 0.5	90.1 ± 1.2	44.2 ± 0.3
2.0	21.9 ± 0.5	12.8 ± 0.3	107.5 ± 3.2	54.3 ± 0.3
4.0	33.3 ± 1.4	13.5 ± 0.7	133.0 ± 5.4	63.2 ± 0.5

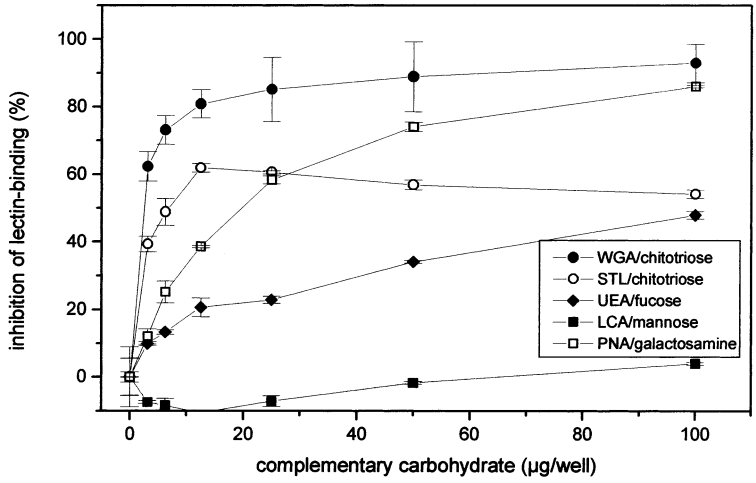

Fig. 3. Competitive inhibition of lectin binding sites on Du-145 cells by addition of the complementary carbohydrate (mean ± S.D., $n = 3$).

were stained with F-WGA and washed to remove unbound lectin. The cells were incubated at 37°C and at regular intervals, up to 2 h the supernatant was assayed fluorimetrically yielding a variation in quantum yield of ± 1.6%. As the F-WGA-content of the supernatant was not altered, the decrease in cell-associated fluorescence intensity by time is not due to dissociation of cell-bound lectin.

3.5. Influence of monensin on fluorescence emission of cell-associated F-WGA

Since the fluorescence emission of fluorescein is known to be reduced in acidic environment, the decreasing quantum yield at 37°C can be due to intracellular uptake of the labeled lectin followed by enrichment in acidic compartments such as lysosomes. Monensin represents a carboxylic ionophore and catalyses the exchange of protons for potassium-ions. Upon treatment of F-WGA-stained cells with monensin, the pH-gradient between the cytoplasm and acidic compartments is compensated.

According to preliminary experiments, up to 20 µM monensin and 10 µl ethanol was well tolerated by Du-145 cells (3.0×10^5 cells) without loss of viability. Additionally the pH-equilibrating effect of the ionophore lasted for about 15 min and decreased afterwards in course of time.

When Du-145 cells preincubated with equal amounts of F-WGA were incubated at 37°C for regular intervals up to 4 h, the cell-associated fluorescence intensity decreased to an extent of 32.2 ± 0.3% ($n = 3$, Fig. 5). But upon addition of monensin, the variation in cell-associated fluorescence intensity was 2.4 ± 0.9% ($n = 15$) only. Consequently, the decreasing quantum yield of cell-associated fluorescein-labeled WGA to an extent of about a third upon incubation at 37°C results from accumulation of the lectin within intracellular acidic compartments.

Table 2
Amounts of complementary carbohydrate as necessary for 50% inhibition of lectin-binding to 3.0×10^5 prostate cancer cells

Lectin/competitive carbohydrate (2 µg/µg)	IC_{50}
WGA/*N*,*N*′,*N*″-triacetylchitotriose	2.5
STL/*N*,*N*′,*N*″-triacetylchitotriose	6.9
UEA-I/fucose	>100.0
PNA/galactosamine	19.7
LCA/mannose	>400.0
DBA/*N*-acetylgalactosamine	No inhibition

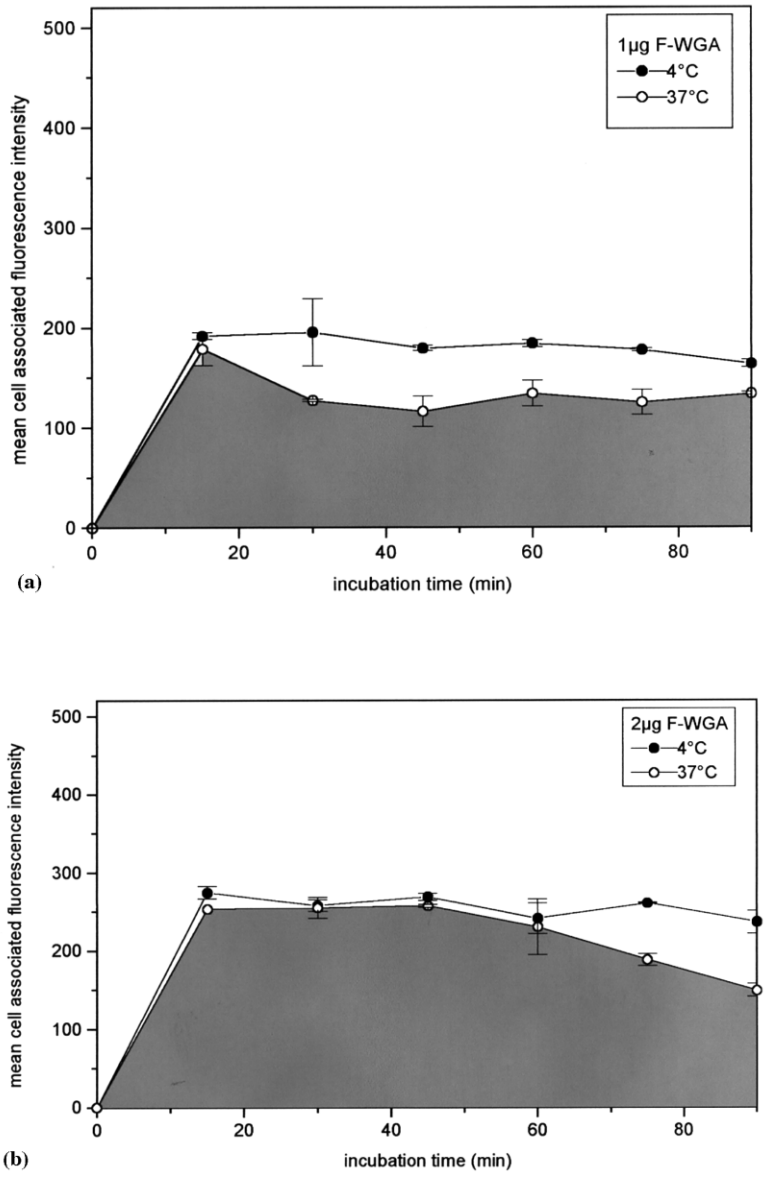

Fig. 4. Amount of Du-145 associated fluorescein-labeled wheat germ agglutinin by course of time at 4 and 37°C, respectively (mean $\pm$ S.D., $n = 3$).

When the same assay was performed at 4°C, the mean cell-associated fluorescence intensity was 390.5 ± 3.5 ($n = 15$) in absence of monensin and 378.0 ± 5.3 ($n = 15$) in presence of monensin. Since addition of monensin decreased the quantum yield of cell-associated F-WGA by 3% only, there is no considerable influence of the ionophore at 4°C. This indicates for preferred binding of labeled lectin to the cell-surface and excludes internalization of the cell-bound lectin at 4°C.

3.6. Confocal laser scanning microscopy of F-WGA stained Du-145 cells

The influence of different temperature levels on cellular distribution of F-WGA was also observed qualitatively by confocal laser scanning microscopy. The images acquired with the focus plane set to the middle of the Du-145 cells incubated with F-WGA at 4°C for 1 h exhibited a fluorescent ring around the cells indicating for membrane-bound lectin, whereas intracellular enrichment of fluorescence was not observed (Fig. 6). When the cells were allowed to interact with equal amounts of F-WGA for the same period of time at 37°C, the fluorescence was found to be located intracellularly. Interestingly, the cytoplasm was not stained uniformly but in a dot like manner indicating for vesicular accumulation of the lectin.

3.7. Effect of ouabain on Du-145 associated F-WGA

When ouabain is present at the extracellular site of the membrane, dephosphorylation of the Na^+/K^{+-} ATPase is impeded followed by inhibition of active transport of substances into the cell.

Upon incubation of cells pretreated with increasing concentrations of ouabain (2.5–40.0 μM) with F-WGA at 4°C, the mean cell associated fluorescence intensity was 183.8 ± 2.5, being comparable to the control without ouabain (176.7 ± 3.2). But at 37°C pretreatment of cells with 10 μM ouabain increased the mean cell associated fluorescence intensity to an extent of 22.2 ± 3.0 as compared with the control. Treating the cells with lower amounts of ouabain resulted in decreasing differences as compared with the control, whereas higher concentrations of ouabain reduced viability of Du-145 cells. Thus the optimum concentration for inhibition of active transport was 10 μM ouabain. The effect of ouabain on cell association of F-WGA was also found to be dependent on time, when the cells were assayed immediately after washing, the mean cell associated fluorescence intensity was 9.5% higher than that of the control, but decreased by time ($+6.7\%$ after 30 min, $+0.6\%$ after 1 h). Additionally the incubation period of Du-145 cells with ouabain prior to addition of WGA strongly influences the results: the effect of ouabain on lectin-transport across the cell membrane decreases with increasing the preincubation time to about a third within 20 min.

As derived from these preliminary assays, the cells were pretreated for 15 min with 10 μM ouabain at 37°C and incubated with F-WGA for 30 min at 37°C. Upon preincubation with ouabain the mean cell associated fluorescence intensity was $21.5 \pm 3.3\%$ (no chase incubation) or

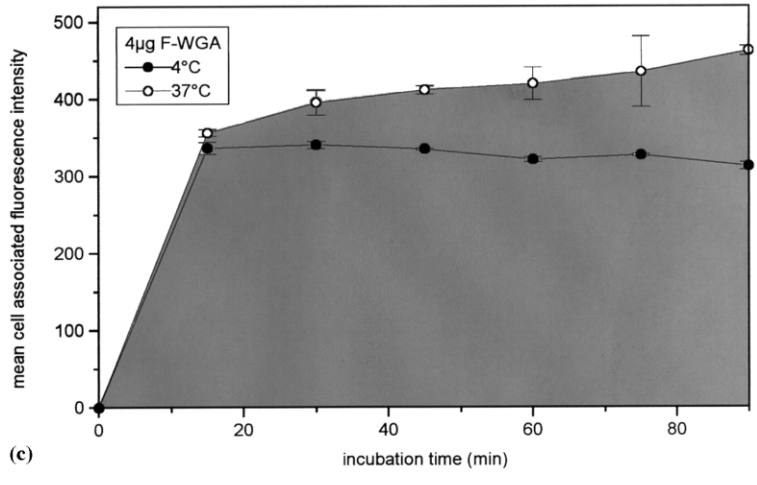

Fig. 4. (*Continued*)

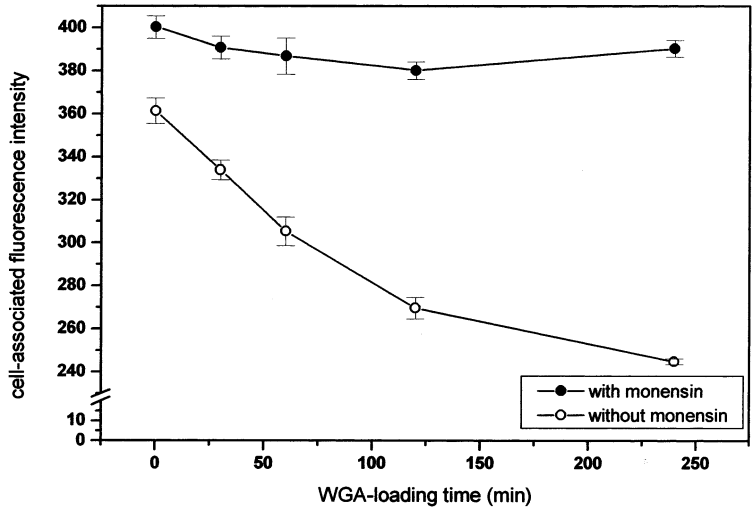

Fig. 5. Cell-associated mean fluorescence intensities after incubation of WGA-loaded Du-145 cells in presence or in absence of monensin

$11.0 \pm 1.6\%$ (chase incubation for 30 min) higher as compared with the control without ouabain (100%, see Table 3). As quenching of the internalized fluorescein-labeled lectin was inhibited by pretreatment with ouabain, these results point to contribution of active transport processes to cellular uptake of membrane-bound WGA.

4. Discussion

To get an idea of the potential utility of lectins as bioadhesive tools for delivery of drugs to prostate cancer cells, the interaction between Du-145 cells and lectins was studied. The cell-association of lectins with different carbohydrate specificities was investigated at both temperature levels, at 4 and 37°C. At 4°C the fluidity of the cell membrane is reduced and energy consuming transport processes are repressed. Consequently, binding of the lectins to the glycocalyx of the prostate cancer cells was determined predominantly. As the lectin-binding rate increased from DBA < PNA < UEA-I < LCA < WGAs < STL to ($\ll$) WGA (Fig. 1), the number of accessible carbohydrates at the surface of Du-145 cells follows the order: sialic acid $\gg$ *N*-acetyl-D-glucosamine > mannose > fucose > galactosamine > *N*-acetyl-galactosamine.

The increase in cell-associated fluorescence intensity concurrent with exposition to increasing amounts of lectins pointed to specificity of the interaction. The specificity of lectin-binding was verified at 4°C following two approaches: first, the amount of cell-bound BSA was attributed to nonspecific binding. Referring the extent of cell-bound BSA to 100% nonspecific binding, contribution of nonspecific protein–membrane interactions to glycocalyx-binding of the lectins under investigation was negligible being 0.05% (WGA), lower than 1.0% (WGAs, LCA, UEA-I, STL) and about 1.5% (PNA, DBA), respectively.

Secondly, specificity was determined by competitive inhibition of the lectin–cell interaction by the corresponding inhibitory carbohydrate. Regardless of the affinity of the lectins to Du-145 cells, the *N*-acetyl-D-glucosamine-binding lectin WGA exhibited highest binding specificity to prostate cancer cells yielding $92.9 \pm 5.7\%$ inhibition of cell-binding upon addition of 100 μg *N*,*N*′,*N*″-triacetylchitotriose per well. The specificity of the other lectins under investigation was found to decrease in the following manner, STL > PNA > UEA-I > LCA. In contrast, the in-

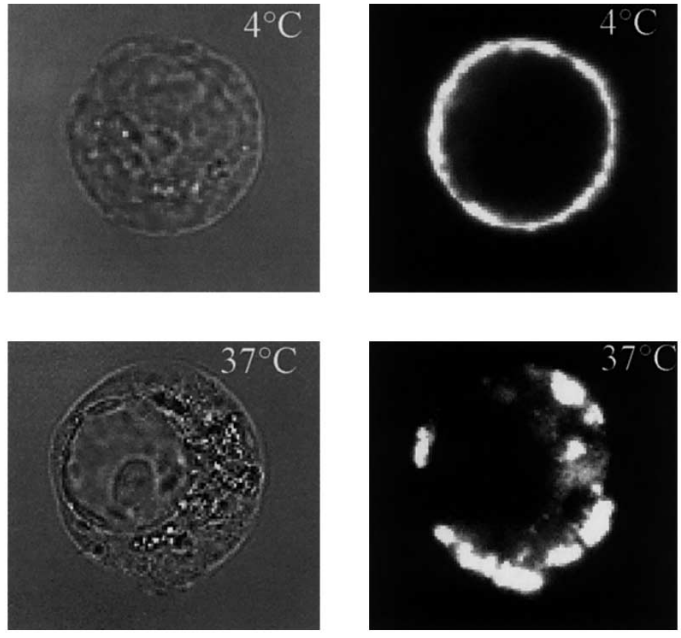

Fig. 6. Confocal images of Du-145 cells stained with fluorescein-labeled WGA for 1 h at 4 or 37°C (left: transmission image, right: flourescence image). The cell diameter refers to about 15 μm.

teraction between DBA and the prostate cancer cells was found to be nonspecific.

Contrary to incubation at 4°C, at 37°C fluidity of the cell-membrane increases and energy-consuming transport processes can occur. Consequently lectin-binding to the cell-membrane can be followed by active transport of the lectin into the cell. Thus the relative fluorescence intensity determined after incubation at 37°C refers to cell-association including binding and uptake of the lectin under investigation. At elevated temperature the order of lectin-association was the same as compared with 4°C incubation, but upon treatment of the cells with comparable amounts of the lectins the mean cell-associated fluorescence strongly decreased (Table 2). This effect amounted to about 50% (WGA and STL at low concentrations), 18–60% (UEA-I), 28–39% (LCA), 22–25% (PNA) or 22–49% (DBA). This decreasing quantum yield might be attributed to different phenomena, (i) dissociation of membrane-bound fluorescent labeled lectin; (ii) intracellular accumulation of initially membrane-bound lectin followed by shielding of the label by cellular components or (iii) cellular uptake of the lectin into acidic compartments such as lysosomes and pH-dependent quenching of the fluorescein label as already observed in the Caco-2 model (Wirth et al., 1998b).

As there was no release of F-WGA from F-WGA stained Du-145 cells even after prolonged incubation, the decreasing quantum yield was not due to dissociation phenomena. Consequently cell-binding of F-WGA does not result from equilibrium between binding and dissociation, but it is a one way process.

Table 3
Cell-associated mean relative fluorescence intensities (mean ± S.D., $n = 3$) of Du-145 cells pretreated with 10 μM ouabain immediately or 30 min after F-WGA loading as compared with the control omitting ouabain-pretreatment

Preincubation	Immediately after F-WGA loading	30 min after F-WGA loading
Without ouabain	148.3 ± 5.7	141.6 ± 0.5
With ouabain	180.2 ± 4.9	157.2 ± 2.2

Intracellular uptake of membrane bound F-WGA into the cytoplasm of Du-145 was confirmed by confocal laser scanning microscopy after incubation at 37°C. In contrast, at 4°C binding to the cell membrane was observed exclusively (see Fig. 6). According to these results the lectin is bound to the cell-membrane upon incubation at 4°C, whereas at 37°C binding is followed by uptake of the lectin into the cells. Thus the lower quantum yield is caused by either shielding effects or fluorescein quenching in acidic intracellular compartments. As uptake occurred at elevated temperature exclusively, involvement of active transport processes was confirmed.

To investigate contribution of Na/K-ATPase on WGA-uptake, experiments in presence of ouabain were performed. Ouabain inhibits transport of compounds into the cytoplasm by inhibition of the Na/K-ATPase. When ouabain-pretreated Du-145 cells were incubated with F-WGA at 37°C, the mean cell-associated fluorescence intensity increased notably as compared with the control without ouabain (Table 3). This exceeding quantum yield indicates that lectin-binding to the membrane is followed by internalization within 30 min. Thus sodium-dependent active transport mechanisms contribute to cytoplasmatic enrichment of the lectin causing a decrease in fluorescence intensity at 37°C. Contrarily upon incubation at 4°C there was no effect of the cardiac glycoside.

As compared with neutral environment the quantum yield of the fluorescein label decreases to about a tenth in acidic milieu. Thus the impact of equilibrating the pH between acidic compartments of the cell and the cytoplasm on cell-associated fluorescence intensity was examined by treatment of F-WGA stained Du-145 cells with monensin. The experiments revealed that the internalized lectin is accumulated in acidic compartments such as the lysosomes or the trans-Golgi network within the cell.

According to the earlier experiments the fluorescein-labeled lectin is specifically bound to the cell-membrane, transported into the cytoplasm and accumulated within acidic compartments. Ongoing from these results the contradictory run of mean cell associated fluorescence intensity by time at 4 or 37°C and different F-WGA concentrations can be explained (Fig. 4). At low concentrations (1 μg F-WGA per well) and 37°C the lectin is bound to the cell-membrane followed by uptake into the cytoplasm within 30 min, but free lectin-binding sites at the cell-surface are not occupied again due to lack of excessive free lectin. Uptake is completed within 30 min, since the mean cell-associated fluorescence intensity nearly remains constant being 127.2 ± 12.5. At medium lectin-concentrations (2 μg F-WGA per well), the mean cell-associated fluorescence intensities at 4 and 37°C are comparable indicative for an equilibrium between binding and uptake of the lectin within 1 h. Within this time interval the recycled binding sites are occupied again due to excess of free lectin. After 1 h of incubation internalization of cell-bound lectin proceeds, but due to lack of excessive free lectin the binding process stagnates causing a decrease in mean cell-associated fluorescence intensity. At high lectin-concentrations (4 μg F-WGA per well) the run of the curves is inverted. At 4°C the binding rate of the lectin to the cell-membrane nearly remains constant due to lacking internalization, whereas at 37°C the lectin-association rate representing the sum of bound and quenched internalized F-WGA increases by time due to availability of free lectin in excess. Comparison of the grey areas under the 37°C curves indicates increasing uptake of cell-bound lectin concurrent with increasing concentration of free available lectin. Since Du-145 uptake of WGA cannot be mediated by binding to immobile *N*-acetyl-D-glucosamine-containing oligosaccharides present at the surface of Du-145 cells, specific carbohydrate-containing receptors are involved in cytoinvasion of the lectin. Radioligand binding studies showed the presence of specific high affinity receptors for epidermal growth factor (EGF) in Du-145 prostate tumors (Lamharzi et al., 1998). Additionally the tyrosine kinase activity of the EGF-receptor isolated from liver was activated by WGA to a similar extent as the enhancement induced by EGF (Zeng et al., 1995). According to these findings, the EGF-receptor might be involved in binding and transport of WGA in prostate cancer cells. In contrast to human fetal prostate and

human benign prostatic hyperbiasia, human adult prostate and human prostate cancer cells were shown to express mRNA-transcripts of the EGF-receptor (Dahiya et al., 1996).

Following on from elucidation of the lectin-binding pattern of prostate cancer cells and characterization of the interaction between WGA and Du-145 cells, especially in view of cytoadhesion and cytoinvasion of WGA, administration of WGA-grafted drug delivery systems might offer an alternative to common prostate cancer therapy such as prostatectomy or i.v.-administration of cytostatic agents being rich in side effects.

References

Dahiya, R., Lee, C., Haughney, P.C., Chui, R., Ho, R., Deng, O., 1996. Differential gene expression of transforming growth factors alpha and beta, epidermal growth factor, keratinocyte growth factor, and their receptors in fetal and adult human prostatic tissues and cancer cell lines. Urology 48, 963–970.

Danguy, A., Decaestecker, C., Genten, F., Salmon, I., Kiss, R., 1998. Applications of lectins and neoglycoconjugates in histology and pathology. Acta Anat. 161, 206–218.

Florence, A.T., Hillery, A.M., Hussain, N., Jani, P.U., 1995. Factors affecting the oral uptake of polystyrene nanoparticles: histological and analytical evidence. J. Drug Targeting 3, 65–70.

Gabor, F., Wirth, M., Walcher, O., Hamilton, O., 1997. Lectin-mediated bioadhesion: gastrointestinal stability and binding-characteristics of *Wheat germ* agglutinin and *Solanum Tuberosum* lectin on Caco-2, HT-29 and human colonocytes. J. Contr. Rel. 49, 27–37.

Gabor, F., Stangl, M., Wirth, M., 1998. Lectin-mediated bioadhesion: binding characteristics of plant lectins on the enterocyte-like cell lines Caco-2, HT-29 and HCT-8. J. Contr. Rel. 55, 131–142.

Jepson, M.A., Mason, C.M., Clark, M.A., Simmons, N.L., Hirst, B.H., 1995. Variations in lectin binding properties of intestinal M cells. J. Drug Targeting 3, 75–77.

Lehr, C.M., 2000. Lectin-mediated drug delivery: the second generation of bioadhesives. J. Contr. Rel. 65, 19–29.

Liener, I.E., Sharon, N., Ooldstein, I.J., 1986. The Lectins: Properties, Functions and Applications in Biology and Medicine. Academic Press, Orlando, FL, pp. 103–115.

Mickey, D.D., Stone, K.R., Wunderli, H., Mickey, O.H., Vollmer, R.T., Paulson, D.F., 1977. Heterotransplantation of a human prostatic adenocarcinoma cell line in nude mice. Cancer Res. 37, 4049–4058.

Peumans, W.J., Van Damme, E.J.M., 1995. Lectins as plant defense proteins. Plant Physiol. 109, 347–352.

Lamharzi, N., Halmos, O., Jungwirth, A., Schally, A.A., 1998. Decrease in the level and mRNA expression of LH-RH and EGF-receptors after treatment with LH-RH antagonist cetrorelix in Du-145 prostate tumor xenografts in nude mice. Int. J. Oncol. 13, 429–435.

Stone, K.R., Mickey, D.D., Wunderli, H., Mickey, O.H., Paulson, D.F., 1978. Isolation of a human prostate carcinoma cell line (DU 145). Int. J. Cancer 21, 274–281.

Wirth, M., Hamilton, G., Gabor, F., 1998a. Lectin-mediated drug targeting: quantification of binding and internalization of wheat germ agglutinin and *Solanum tuberosum* lectin using Caco-2 and HT-29 cells. J. Drug Targeting 6, 95–104.

Wirth, M., Fuchs, A., Wolf, M., Ertl, B., Gabor, F., 1998b. Lectin-mediated drug-targeting: characteristics and antiproliferative activity of wheat germ agglutinin conjugated doxorubicin on Caco-2 cells. Pharm. Res. 15, 1031–1037.

Zeng, F.Y., Benguria, A., Kafert, S., André, S., Gabius, H.J., Villalobo, A., 1995. Differential response of the epidermal growth factor receptor tyrosine kinase activity to several plant and mammalian lectins. Mol. Cell Biochem. 142, 117–124.