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Lectin-mediated drug delivery: binding and uptake of BSA-WGA conjugates using the Caco-2 model

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Abstract

To examine whether the dietary lectin wheat germ agglutinin (WGA) can facilitate binding and uptake of protein drugs due to its cytoadhesive and cytoinvasive properties, conjugates were prepared by covalent coupling of fluorescein-labeled bovine serum albumin (F-BSA) to WGA using divinylsulfone for crosslinking. Increasing the molar ratio of F-BSA/WGA resulted in 2.6–8.7 times higher Caco-2 binding as compared with glycyl-F-BSA. About 75% of F-BSA-WGA were bound specifically to Caco-2 cells according to inhibition studies in presence of the complementary carbohydrate. The Caco-2 association of F-BSA-WGA was temperature-dependent indicating active uptake of membrane bound conjugate, which was confirmed by confocal microscopy. The conjugate accumulated within lysosomal compartments followed by proteolytic degradation of F-BSA-WGA 1–4 h after conjugate loading as observed by equilibrating the intracellular pH with monensin. Finally low molecular weight degradation products of the proteinaceous prodrug appear in the extracellular medium. Contrary to Caco-2 single cells, a minor part of the conjugate is degraded by brush border proteases already 30 min after exposure to Caco-2 monolayers. But most of the conjugate is taken up into differentiated cells and processed as in single cells. Though the enzymic barrier remains to be surmounted, WGA-mediated drug delivery is a promising strategy for peroral delivery of even high molecular weight drugs to overcome the mucosal barrier.

Keywords: Active transport; Caco-2; Lectin; Mucosal barrier; Monensin; Protein drug delivery; Wheat germ agglutinin

1. Introduction

Recent advances in molecular biology and biotechnology stimulated considerable interest in establishing peptides and proteins as drugs in therapy. To date several challenges confront the peroral delivery of peptide and protein drugs. First preservation of structural integrity until reaching the site of action is required, secondly closely related to the first degradation by the enzymatic barrier is another challenge. Thirdly, the resistance of the mucosal barrier to penetration by protein drugs has to be surmounted (Lee, 1991).

To achieve specific delivery of proteins and peptides across the intestinal epithelium after peroral administration receptor—mediated endocytosis can be utilized as a pathway. Recent

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investigations have focused on drugs conjugated with vitamins, bile acids and transferrin.

The covalent coupling of the vitamin folic acid via its γ-glutamyl carboxylate to chemotherapeutic agents, radiopharmaceuticals, nanoparticles and DNA transfection complexes yields conjugates that can be endocytosed into folate-receptor bearing cells. As folate receptors are significantly overexpressed on a high number of human tumors, endocytosis of folate-conjugates is an encouraging strategy to tumor targeted drug delivery (Wang and Low, 1998; Leamon and Low, 2001). Utilizing the ileal vitamin B12 uptake system represents another concept to enhance the absorption of protein and peptide pharmaceuticals including erythropoietin and granulocyte colony stimulating factor (Habberfield et al., 1996). The carrier capacity of the vitamin B12 receptor is rather low being about 1 nmol per dose in humans, but it might be enhanced by use of vitamine B12 grafted nanoparticles (Russell-Jones et al., 1999). Additional problems can arise from interference of vitamin B12 mediated uptake with the absorption of nutrients (Lavelle, 2000).

Utilization of body's own carrier systems for transmembrane transport of peptides also includes the intestinal bile acid transporter, which facilitates a daily absorption of 10–20 g of bile acids. Cholic acid–peptide conjugates were shown to be transported across Caco-2 monolayers involving the carrier mediated pathway in case of conjugating peptides composed of up to four amino acids. The enhanced transport rate of conjugates with six amino acids was attributed rather to increased passive membrane permeability of the conjugates than to the carrier-mediated pathway (Swaan et al., 1997).

Another strategy to enhance absorption of proteins relies on exploiting the pathway of iron-absorption. Besides catalase (Rojanasakul et al., 1996) and saporin (Ippoliti et al., 1995), in particular a lot of work concentrates on peroral delivery of insulin by transferrin-induced endocytosis. As the transferrin receptor is essentially localized on the basolateral membrane of Caco-2 cells and enterocytes (Banerjee et al., 1986; Fuller and Simons, 1986), co-administration of the GTPase inhibitors brefeldin A or tyrphostin-8 is required to enrich sufficient numbers of transferrin receptors at the apical membrane in order to promote GI-absorption of insulin-transferrin conjugates (Shah and Shen, 1996; Xia and Shen, 2001). Even in vivo a slow and prolonged hypoglycemic effect was observed in diabetic rats after peroral administration of appropriate insulin-transferrin conjugates (Xia et al., 2000).

Moreover, there is evidence from the literature, that some plant lectins can facilitate the transport across cellular barriers. One hour after ingestion of 200 g of raw or roasted peanuts by healthy volunteers up to 5 μg/ml intact peanut lectin were detected in serum (Wang et al., 1998). Tomato lectin is transported across intestinal mucosa by endocytosis in vitro, but uptake in vivo was circumstantial due to mucus-binding. However, conjugation might enhance bioavailability of poorly absorbable drugs as very low doses of ingested tomato lectin were immunogenic in humans (Woodley, 2000).

Wheat germ agglutinin (WGA) binds to *N*-acetyl-D-glucosamine and sialic acid exhibiting a molecular weight of 36 kDa. As compared with plant lectins with different carbohydrate specificity, the WGA-binding rate to intestinal cell lines of human origin, human colonocytes and prostate cancer cells was highest (Gabor et al., 1997, 1998, 2001). Moreover, the WGA not only binds to the cell membrane, but it is also taken up into the cytoplasm of enterocyte-like Caco-2 cells (Wirth et al., 1998).

Thus the objective of this work is to get evidence whether WGA can facilitate the transport of conjugated high molecular weight compounds into Caco-2 cells. To follow this approach, fluorescein labelled bovine serum albumin (F-BSA) was used as a model protein, which was covalently bound to WGA by divinyl sulfone.

2. Materials and methods

2.1. Chemicals

WGA from *Triticum vulgare* was obtained from Vector laboratories (Burlingham, USA). F-BSA, divinylsulfone, glutardialdehyde, fluorescein, *N*,

N′,*N*″-triacetylchitotriose, *N*-acetyl-D-galac tosamine and dextran blue were purchased from Sigma (St. Louis, MO, USA). Superdex 200 prep grade from Pharmacia Biotech (Uppsala, S) was used for gel permeation chromatography. Ultrafree MC–filters (Amicon, Beverly, MA, USA) were applied to concentrate the conjugates. SDS-PAGE was performed using a Mini-Protean II apparatus and a broad range marker from Bio-Rad (California, USA). All other chemicals were of analytical grade and obtained from Merck (Darmstadt, G).

2.2. Cell culture

The Caco-2 cell line was purchased from the American Type Culture Collection (Rockville, MD, USA) and used between passage 35 and 50; tissue culture reagents were from Sigma and Gibco Life Technologies Ltd. (UK).

Caco-2 cells were grown in RPMI 1640 cell culture medium containing 10% fetal calf serum, 4 mM L-glutamine and 150 μg/ml gentamycine in a humidified 5% CO_2/95% air atmosphere at 37 °C and subcultivated by trypsination.

For binding assays, cells were seeded on TC-treated 96-well microplates (Greiner, Kremsmünster, A) at a density of 1.7×10^4 per well. The cells were fed every other day with culture medium and used between day 12 and 14 after seeding.

2.3. Preparation of F-BSA-WGA conjugates

All reactions were carried out under protection from light to prevent bleaching of the fluorescein label. The primary amino residues of F-BSA were activated by adding dropwise a solution of 0.5 mg F-BSA (7.6 nmol) in 0.5 ml 20mM HEPES/NaOH buffer pH 8.0 to a mixture of 0.1 ml divinyl sulfone and 0.4 ml dioxane. After stirring for 1 h at room temperature excessive divinyl sulfone was removed by exhaustive dialysis against 20 mM HEPES/NaOH buffer pH 8.0. The lectin was linked covalently to the vinyl sulfone derivative of F-BSA using a 2.5-, 5-, 10- and 20-fold molar excess of WGA as compared with F-BSA, respectively. For this purpose WGA was dissolved in 1 ml 20mM HEPES/NaOH buffer pH 8.0, mixed with the solution containing activated F-BSA and rotated end over end for 20 h at room temperature. Non-reacted vinyl groups were blocked by addition of glycine and further incubation for 1 h.

The F-BSA-WGA conjugates were fractionated and purified by gel filtration chromatography on Superdex 200 columns (1×40 cm) by elution with isotonic 20 mM HEPES/NaOH buffer pH 7.4 at a flow rate of 0.8 ml/min. The fraction size was 650 μl. F-BSA as well as the conjugates were detected by fluorescence emission of their fluorescein label.

F-BSA treated as described above but omitting addition of WGA served as a control and corresponds to glycyl-F-BSA.

2.4. Estimation of the molecular weight of the F-BSA-WGA conjugates

The molecular weight of the conjugates was determined by gel filtration on Superdex 200 and electrophoresis. Dextran blue, F-BSA, F-WGA and fluorescein were used for calibration of the column and the experiment was performed as described above.

Prior to 10% SDS-PAGE (Laemmli, 1970) the F-BSA-WGA conjugates were concentrated by use of Ultrafree MC—filters in as much as the conjugates were above the detection limit of the Bradford test (Bradford, 1976). F-BSA, WGA and the broad range marker were used as marker proteins to establish the calibration curve for determination of the molecular weight of the conjugates.

2.5. Interaction of the F-BSA-WGA conjugates with Caco-2 cells

Binding of the conjugates to the surface of Caco-2 cells was investigated using both single cells and monolayers.

To assess the interaction between F-BSA-WGA and Caco-2 single cells 50 μl of a solution containing 0.4 μg F-BSA in isotonic 20 mM HEPES/NaOH buffer pH 7.4 were incubated with 50 μl cell suspension (3×10^5 cells) for 20 min at 4 °C. After removal of unbound conjugate by washing

the cells two times with 150 μl of the same buffer the cell-bound fluorescence intensity was determined by flow cytometry.

Each test was performed twice at the minimum. Negative controls omitting addition of the conjugate were included in every experiment and substracted from all binding data quoted.

To estimate the binding rate of F-BSA-WGA on monolayers Caco-2 cells were grown in 96-well microplates for 12–14 days. Prior to the binding assay the cell layers were washed twice with 100 μl isotonic 20 mM HEPES/NaOH buffer pH 7.4 followed by incubation with 50 μl of a solution of 2 μg F-BSA-WGA in isotonic 20 mM HEPES/NaOH buffer pH 7.4 for 20 min at 4 °C. After determination of the relative fluorescence intensity (RFI) unbound F-BSA-WGA was removed by washing the Caco-2 monolyers two times with the same buffer. The cell-bound fluorescence intensity was monitored using a fluorescence microplate reader (Spectrafluor Fluorometer, TECAN, Grödig/Salzburg, A) at 485/535 nm.

Tests were carried out in triplicate and repeated at least twice. RFI values after incubation with buffer only served as a negative control to estimate the autofluorescence of the plate and the cells. These values were subtracted from all binding data quoted. The binding rate was calculated from the quotient of cell-bound F-BSA-WGA and total amount of F-BSA-WGA added.

For both assays, the binding rate of comparable amounts of glycyl-F-BSA was determined for comparison.

2.6. Flow cytometry

Flow cytometric measurements were performed on an EPICS XL-MCL analytical flow cytometer (Coulter, Miami, USA). The labeled cell suspension was resuspended in 1 ml Cell Pack prior to the measurement. Cells were analyzed using a forward versus side scatter gate for inclusion of the single cell population and exclusion of debris and cell aggregates. Fluorescence was detected at 525 nm (10 nm bandwidth) and the mean channel number of the logarithmic intensities of individual peaks was used for further calculations. Amplification of the fluorescence signals was adjusted to put the autofluorescence signal of unlabelled cells in the first decade of the four-decade log range. For each measurement data of 3000 cells were accumulated.

2.7. Specificity of the binding of F-BSA-WGA to Caco-2 cells

Carbohydrate specificity of the interaction between the F-BSA-WGA conjugates and Caco-2 cells was assessed by a competitive assay using *N*,*N*′,*N*″-triacetylchitotriose. 50 μl Caco-2 cell suspension (3×10^5 cells), 50 μl of a solution of F-BSA-WGA corresponding to 2 μg F-BSA in isotonic 20 mM HEPES/NaOH buffer pH 7.4 and 100 μl of a solution of *N*,*N*′,*N*″-triacetylchitotriose in the same buffer (1, 0.75, 0.5, 0.25, 0.1 mg/ml) were mixed and incubated for 20 min at 4 °C. Cells were spun down (1000 rpm, 5 min, 4 °C) and 150 μl of the supernatant were discarded. After addition of 150 μl buffer, the washing step was repeated in the same manner prior to flow cytometry.

Each concentration was analyzed in triplicate. Negative controls were included in every experiment and carried out as described above. Using isotonic 20 mM HEPES/NaOH buffer pH 7.4 instead of the carbohydrate solution served as a positive control representing the maximum amount of Caco-2 bound conjugate.

2.8. Confocal laser scanning microscopy

To investigate the uptake of F-BSA-WGA into Caco-2 cells 50 μl cell suspension (2×10^5 cells) were incubated with 50 μl of a solution of F-BSA-WGA (35 μg/ml isotonic 20 mM HEPES/NaOH buffer pH 7.4) for 1 h at 4 and 37 °C, respectively. Cells were collected by centrifugation and washed twice as described above prior to mounting for microscopy. Confocal images of fluorescent labeled cells were obtained using a Zeiss Axiovert confocal microscope. Transmission light and fluorescence pictures were acquired at a magnification of 40× and the black level (background offset) of the green fluorescence detector was adjusted to eliminate any autofluorescence of unstained cells. Cell associated F-BSA either free

or conjugated was detected by excitation at 488 nm and emission > 515 nm.

2.9. Internalization and intracellular distribution of F-BSA-WGA

To study the uptake and intracellular accumulation of the conjugate after binding to the surface of Caco-2 cells the interaction between F-BSA-WGA and Caco-2 cells was examined at different temperature levels. Additionally the influence of monensin on fluorescence intensity deriving from cell associated F-BSA-WGA was investigated.

About 50 μl of a solution containing F-BSA-WGA in isotonic 20 mM HEPES/NaOH buffer pH 7.4 (35 μg/ml) and 50 μl Caco-2 cell suspension (3×10^5 cells) were incubated at 4 °C for 20 min to bind the conjugate to the cell surface. To estimate potential uptake of the cell bound conjugate the cells were washed as described above to remove any unbound conjugate. After adjusting the volume to 100 μl the loaded cells were incubated at either 4 or 37 °C for 15–360 min. Cells were washed again and cell-associated F-BSA-WGA was analyzed by flow cytometry.

The accumulation within acidic compartments of the cell was assessed by incubating the cells for 15–360 min at 37 °C after a puls incubation with the F-BSA-WGA conjugate (35 μg/ml) for 20 min at 4 °C. Cells were washed and analyzed flow cytometrically. After addition of 10 μl of a solution of monensin (2.42 μmol/ml ethanol) and incubation for 3 min at room temperature the cell-associated fluorescence intensity was measured again by flow cytometry.

Experiments using Caco-2 monolayers were performed with cell layers grown for 12–14 days. The monolayers were preincubated with 50 μl F-BSA-WGA solution (35 μg/ml isotonic 20 mM HEPES/NaOH buffer pH 7.4) for 20 min at 4 °C. After washing the monolayers were incubated for 10–300 min at 37 °C followed by assessment of the total fluorescence intensity of the cell layers. The monolayers were washed twice as described above and the cell-associated fluorescence intensity was monitored. After removal of the supernatant the monolayers were incubated with 50 μl of a solution of monensin (21.8 nmol/ml isotonic 20mM HEPES/NaOH buffer pH 7.4) for 3 min at room temperature and the cell associated fluorescence intensity was determined again.

Negative controls were included in every experiment and treated as described above.

3. Results

3.1. Preparation of F-BSA-WGA conjugates

Divinyl sulfone, which permits conjugation of either amino-, hydroxyl- or sulfhydryl groups depending on the coupling pH, was used for covalent conjugation of the lectin WGA to F-BSA as a fluorescent model protein under mild conditions (Houen and Jensen, 1995; Fig. 1). To minimize loss of lectin-activity, amino-residues of F-BSA were activated with divinyl sulfone and excess of crosslinker was removed by exhaustive dialysis. At slightly alkaline pH activated F-BSA was reacted with free accessible amino groups of WGA yielding stable amine bonds. To avoid crosslinking of the parent compounds and formation of uneven substituted conjugates a two step mechanism was pursued. The glycine-saturated F-BSA-WGA conjugates were purified by gel filtration chromatography. Independent from the molar ra-

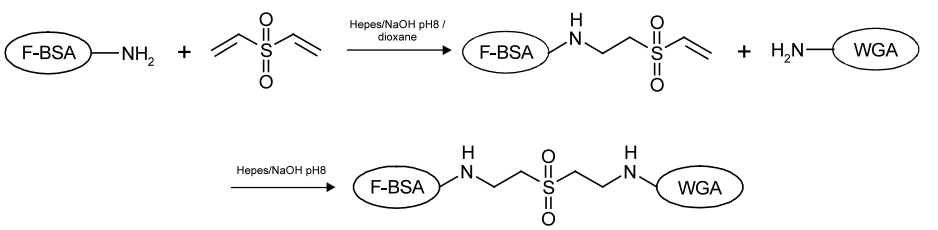

Fig. 1. Reaction scheme of covalent coupling of F-BSA to WGA by divinyl sulfone.

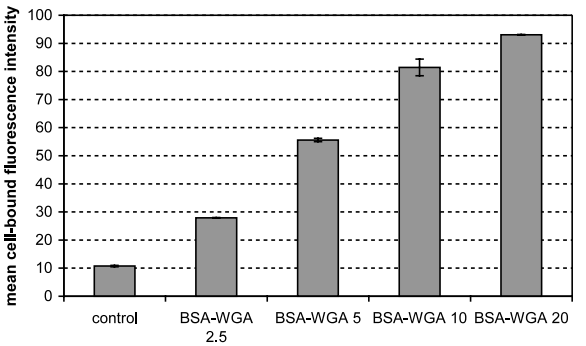

Fig. 2. Caco-2 binding of F-BSA-WGA conjugates prepared at different molar ratio of F-BSA/WGA (1:2.5, BSA-WGA2.5; 1:5, BSA-WGA5; 1:10, BSA-WGA10; 1:20, BSA-WGA20) at 4 °C (mean ± S.D., $n = 3$).

tio of F-BSA and WGA ranging from 1:2.5 to 1:20 two peaks of each conjugate were detected at an elution volume of 18.2 and 21.4 ml. As the parent compound of higher molecular weight (F-BSA, Mw 66 kDa) was eluted at 22.4 ml, this indicates for covalent binding of WGA to F-BSA upon conjugation.

3.2. Caco-2 binding of F-BSA-WGA conjugates

In order to investigate the influence of increasing the molar ratio of F-BSA and WGA on Caco-2 binding of the purified conjugates, the cells were allowed to interact for 20 min at 4 °C with the conjugates prepared from F-BSA and WGA at a molar ratio of 1:2.5 (BSA-WGA2.5), 1:5 (BSA-WGA5), 1:10 (BSA-WGA10), 1:20 (BSA-WGA20). As determined by flow cytometry, the Caco-2 binding rate of the conjugates increased concurrent with increasing the amount of WGA used for conjugation procedure (Fig. 2). Compared with the control without WGA representing glycine-modified F-BSA, the interaction with Caco-2 single cells was 2.6 times higher with the conjugate BSA-WGA2.5 and even up to 8.7 times higher in case of the conjugate prepared with the highest amount of WGA (BSA-WGA20).

The purification of each of the conjugates yielded two peak fractions, but resolution of the gel filtration medium was not sufficient in that the molecular weight could be determined exactly. Thus the peak fractions of the BSA-WGA20 were collected and analyzed by SDS-PAGE. In comparison to F-BSA (Mw 66 kDa) and WGA (Mw_{dimer} 36 kDa, $Mw_{monomer}$ 18 kDa), the conjugate eluted at the lower retention volume of 18.2 ml exhibiting a molecular weight of 200–250 kDa (BSA-WGA20a). In contrast, the protein peak eluted at a retention volume of 21.4 ml contained conjugates with a molecular weight of about 80 kDa (BSA-WGA20b).

According to the difference in molecular weight it seemed likely that the conjugate fractions differ in their degree of lectin substitution. Thus the binding characteristics of BSA-WGA20a and BSA-WGA20b were investigated using Caco-2 monolayers grown in 96 well microplates for at least 12 days. After incubation with the F-BSA-WGA20 conjugate fractions (35 μg/ml) for 20 min at 4 °C about 18.5 ± 1.0% of the higher molecular weight conjugate BSA-WGAa were bound to the cell surface. In case of the 80 kDa-conjugate (BSA-WGA20b) 19.6 ± 1.7% adhered to the cell surface, whereas only 1.0 ± 0.73% of glycyl-BSA were bound to the cells. These results indicate that the bound fraction of the conjugates is rather independent from the size of the conjugate within this unknown degree of lectin-substitution.

3.3. Specificity of F-BSA-WGA binding to Caco-2 cells

To estimate the extent to which Caco-2 binding of BSA-WGA occurs via the carbohydrate moieties of the glycocalyx at the cell surface a competitive assay was performed using *N*,*N*′,*N*″-triacetyl-chitotriose to inhibit the interaction between the conjugates and the cells. When Caco-2 cells were allowed to interact with a solution containing the F-BSA-WGA20 conjugate (F-BSA-content: 2 μg) and the complementary carbohydrate at 4 °C, the amount of cell-bound conjugate decreased with increasing carbohydrate concentrations (Fig. 3). Interestingly, the extent of inhibition was different in case of the conjugates BSA-WGA20a and BSA-WGA20b within the range of 0.1 up to 0.75 mg/ml carbohydrate yielding an inhibition of 32.4 ± 0.7% (BSA-WGA20a) or 49.3 ± 1.5% (BSA-WGA20b) at an inhibitor

concentration of 0.25 mg/ml. This diversity in the run of the curves points to different specificity of the interaction between the cell surface and the conjugates depending on the size of the molecule being 1.5 times higher in case of the low molecular weight conjugate. In presence of high amounts of carbohydrate Caco-2 binding of the conjugates was inhibited to an extent of $76.1 \pm 1.5\%$ (BSA-WGA20a) and $76.0 \pm 0.6\%$ (BSA-WGA20b), respectively.

Since specificity of adhesion to Caco-2 cells is higher in case of the low molecular weight conjugate and cellular uptake is probably better with smaller molecules, further characterization of the F-BSA-WGA conjugates in terms of internalization and intracellular distribution was performed using BSA-WGA 20b.

3.4. Influence of temperature on Caco-2 association of the F-BSA-WGA conjugate

To guarantee comparability of the results Caco-2 single cells were loaded with F-BSA-WGA20b for 20 min at 4 °C followed by incubation at either 4 or 37 °C up to 360 min. Upon incubation at 4 °C the cell-associated fluorescence intensity did not vary markedly indicating that initially cell-bound conjugate was not detached during incubation (Fig. 4).

At 37 °C the cell-associated fluorescence intensity decreased with incubation time to 65% within 60 min, but fluorescent compounds could not be detected in the supernatant. After 1 h of incubation at 37 °C the decrease of the cell-associated fluorescence signal was inverted into an increase. For comparison, the Caco-2 associated fluorescence intensity after incubation with glycyl-F-BSA was only 1.1 ± 0.1.

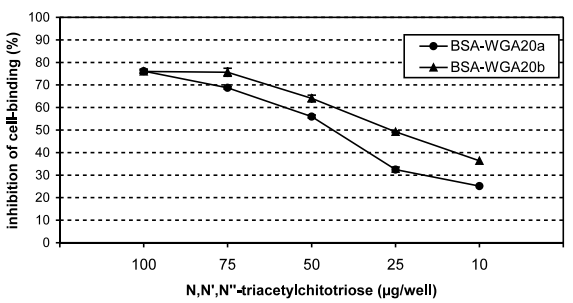

Fig. 3. Competitive inhibition of F-BSA-WGA20a (Mw 200–250 kDa) and F-BSA-WGA20b (Mw 80 kDa) binding to Caco-2 single cells by addition of the complementary carbohydrate (mean $\pm$ S.D., $n = 3$).

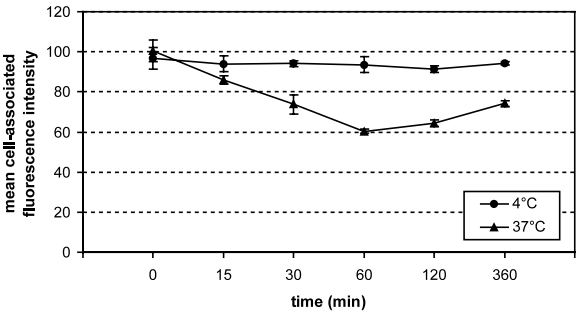

Fig. 4. Mean Caco-2 associated fluorescence intensity deriving from F-WGA-BSA20a (Mw 200–250 kDa) after incubation at 4 and 37 °C, respectively (mean $\pm$ S.D., $n = 3$).

3.5. Confocal laser scanning microscopy of conjugate loaded Caco-2 cells

To gain more information about the influence of temperature on the fate of the conjugate resulting from flow cytometry-experiments at 4 and 37 °C Caco-2 cells were incubated with the F-BSA-WGA20b conjugate at both temperature levels for 60 min followed by CLSM analysis.

After incubation at 37 °C a dot-like pattern of the fluorescence around the nucleus originating from the fluorescent BSA-moiety of the conjugate was observed (Fig. 5). This pointed to uptake and vesicular accumulation of the conjugate within the Caco-2 cell. In contrast, at 4 °C a fluorescent ring around the cell indicated for solely binding of the conjugate to the glycocalyx at the cell surface when the focus plane was set to the middle of the cell. When the cells were incubated with glycyl-F-BSA, neither binding to the cell-membrane nor uptake into the cells was observed.

3.6. Influence of monensin on the fluorescence of single cell associated conjugate

Since at the one hand the conjugate is taken up into the cells according to the CLSM and on the

other hand the quantum yield of the conjugate is reduced at 37 °C as compared with 4 °C, these arbitrary contradictory effects should be clarified by experiments at 37 °C followed by addition of monensin. Monensin acts as a carboxylic ionophore catalyzing the exchange of protons for potassium-ions. Upon treatment with monensin the pH-gradient between acidic intracellular compartments and the cytoplasm is compensated. Thus abolishment of the quench of the fluorescein-label, which is known to occur in acidic milieu, should raise the quantum yield of F-BSA-WGA in case of uptake of the conjugate into the Caco-2 single cells.

As described above Caco-2 cells were loaded with the fluorescein labeled conjugate at 4 °C followed by incubation for up to 6 h at 37 °C. In accordance with previous results (Fig. 4) in absence of monensin the cell bound fluorescence intensity decreased by time to an extent of 52.9 ± 0.9%, whereas the fluorescence signal after addition of monensin was not altered notedly within 60 min (Fig. 6). These results point to rapid internalization of the BSA-WGA conjugate at 37 °C followed by distribution to acidic compartments of the cell. After 1 h of incubation both the emission signal prior to and after monensin addition increased with prolonged incubation. Incubating Caco-2 cells with the BSA-WGA conjugate

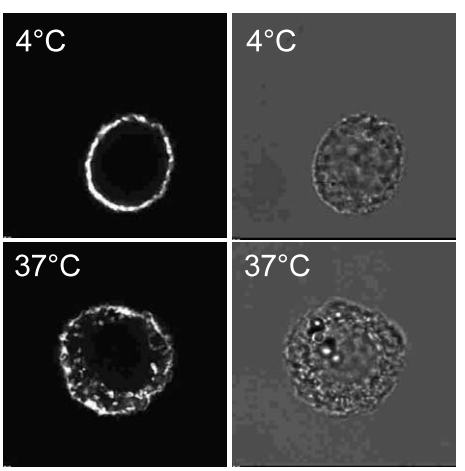

Fig. 5. Confocal images of Caco-2 cells stained with F-BSA-WGA20a for 1 h at 4 or 37 °C (left, flourescence image; right, transmission image). The cell diameter refers to about 15 μm.

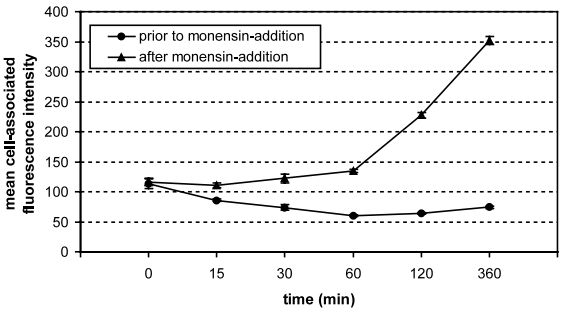

Fig. 6. Mean Caco-2 associated fluorescence intensities of F-BSA-WGA20a-loaded single cells prior and after addition of monensin by time up to 6 h (mean ± S.D., $n = 3$).

for 6 h resulted in a non-quenched cell associated fluorescence signal being more than three times higher than the initial emission of membrane bound conjugate. Probably intravesicular proteolytic degradation of the conjugate resulted in formation of low molecular weight compounds exhibiting reduced quenching as compared with the parent high molecular weight conjugate. But in absence of monensin only a moderate increase of signal intensity was observed. This might be due to a limited number of fluorescent degradation products outside the lysosomes within this observation period.

In order to get an idea of the cellular fate of the conjugate by time, the experiment was repeated, but incubation was extended to 18 h and the supernatant of the cells was analyzed fluorimetrically. In presence of monensin the non-quenched cell associated fluorescence intensity increased continuously up to 5 h (Fig. 7). Within this time interval not any fluorescence was detected in the supernatant. Upon further incubation the cell associated fluorescence intensity decreased concurrent with increasing fluorescence intensity of the supernatant. After 18 h of incubation the cell associated fluorescence intensity decreased to about a half compared with 5 h incubation. Concurrently the fluorescence intensity of the supernatant was more than 30 times higher than that after 5 h. This decrease of cell-associated fluorescence intensity might be due to excretion of initially cell associated conjugate or fluorescent degradation products of the F-BSA-WGA as indicated by the increasing fluorescence signal in the supernatant.

3.7. Influence of monensin on the fluorescence of monolayer associated conjugate

In contrast to Caco-2 single cells, Caco-2 cells forming a monolayer undergo a differentiation process after reaching confluency. Thus experiments were performed as above, but using Caco-2 monolayers grown for at least 12 days. Contrary to single cells, formation of an artificial intestinal tissue resulted in a strongly different run of fluorescence intensity deriving from cell-associated F-BSA-WGA (Fig. 8). In accordance with the single cell experiments the mean cell associated fluorescence intensity initially decreased in absence of monensin, but lasting quite longer yielding a loss in fluorescent intensity of about 60% within 3 h. In contrast to single cell experiments the non-quenched cell associated fluorescence intensity decreased too. As compared with the monolayer experiment in absence of monensin the descent of fluorescence emission was more moderate being about 30% within the same time interval. Whereas no fluorescence emission was detectable in the supernatant of single cells up to 5 h, the supernatant of the monolayers contained fluorescent compounds even after 30 min and the fluorescence intensity increased further until the end of the experiment. This initial decrease in cell associated fluorescence intensity in presence of monensin concurrent with an increase of the fluorescence signal in the supernatant might be due to proteolytic degradation of initially membrane bound conjugate by the brush border hydrolases. But the run of descending cell-associated fluorescence intensity prior and after monensin-addition was quite different resulting in a mean cell associated fluorescence intensity of 90.8 ± 31.4 and 161.5 ± 30.5 after 30.5 respectively. This points to uptake and accumulation of the conjugate within acidic compartments of the Caco-2 monolayer. After 3 h both, the cell associated fluorescence intensity in absence and in presence of monensin increased pointing to cleavage of the conjugate within the lysosomes followed by an increase in the quantum yield of the fluorescein label as seen with the single cells. After 5 h of incubation the cell associated fluorescence intensity amounted to 123.2 ± 11.9 in absence of monensin and 220.5 ± 11.9 in presence of monensin.

4. Discussion

Peroral delivery of peptide and protein drugs is still a challenge due to their physicochemical and biological properties, all in all resulting in poor bioavailability. In an effort to develop novel strategies for delivery of proteins, the potential utility of cytoadhesive and cytoinvasive WGA as

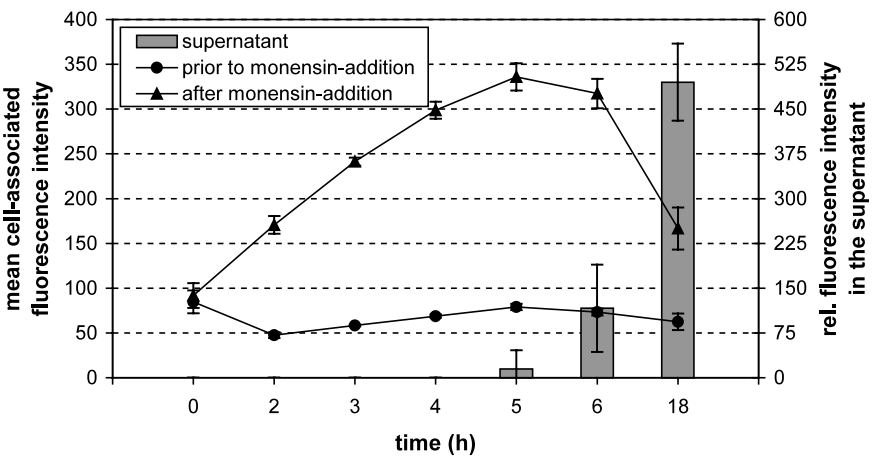

Fig. 7. Mean Caco-2 associated fluorescence intensities of F-BSA-WGA20a-loaded single cells prior and after addition of monensin by time up to 18 h in comparison to the fluorescence intensity of the supernatant (mean $\pm$ S.D., $n = 3$).

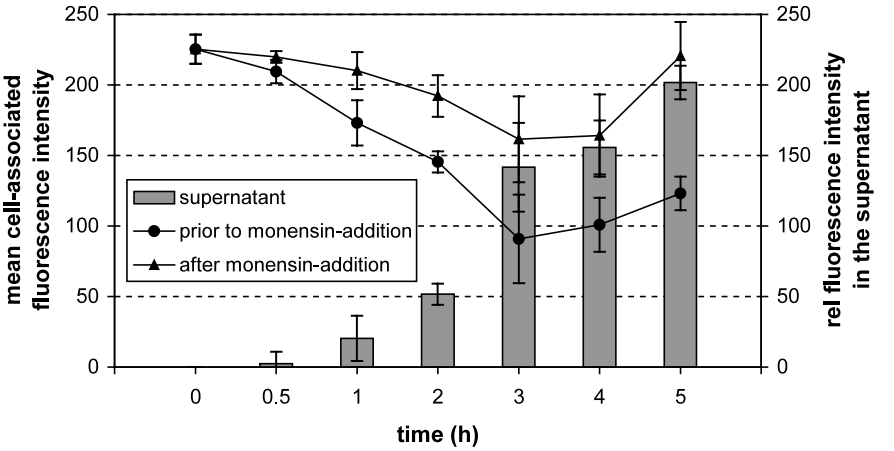

Fig. 8. Caco-2 monolayer associated fluorescence intensities of F-BSA-WGA20a-loaded cells prior and after addition of monensin by time up to 18 h in comparison to the fluorescence intensity of the supernatant (mean ± S.D., $n = 3$).

a 'shoehorn' for improved peroral absorption of proteins was investigated.

The fluorescein-labeled model protein BSA was linked covalently to the lectin WGA by divinylsulfone following a two step mechanism. Successful conjugation was confirmed by gel filtration and SDS-PAGE yielding conjugates of higher molecular weight than the parent compounds. Increasing the molar ratio of F-BSA and WGA during synthesis resulted in conjugates with improved binding to Caco-2 single cells being up to 8.7 times higher than glycine-modified F-BSA. These results indicate that the amount of WGA linked covalently to the model protein is different and depends on the molar ratio between F-BSA and the lectin. But due to the results of the gel permeation chromatography the size of the conjugate molecules was not observed to be altered. According to molecular weight determinations two types of conjugates were obtained independent from the initial molar ratio of F-BSA and WGA exhibiting a molecular weight of 80 and 200–250 kDa (SDS-PAGE), but their bound fraction to Caco-2 monolayers was quite similar. The Caco-2 monolayer binding of 19% of the conjugate F-BSA-WGA20 as opposed to 1% in case of glycyl-F-BSA confirms improved binding of the model drug due to conjugation with the lectin.

Specificity of the Caco-2–conjugate interaction was assessed by a competitive assay in presence of increasing amounts of the free carbohydrate complementary to the lectin binding site. According to the results, about 75% of F-BSA-WGA were bound specifically involving the carbohydrate binding sites of the conjugated lectin. Whereas only 2.7% of the F-WGA–Caco-2 interaction were due to non-specific binding (Wirth et al., 1998), this amount increased to 25% by coupling of F-BSA. Nevertheless the lectin binding site seems to remain unaffected by the coupling procedure.

WGA was found not only to adhere to the glycocalyx of Caco-2 cells, but also to be taken up into the cells (Wirth et al., 1998). To examine whether the conjugates are taken up into the cell due to their lectin content, Caco-2 association assays were performed at different temperature levels. At 4 °C the metabolism of the cells is reduced to a minimum and active transport processes are restricted. Thus the cell-associated fluorescence intensity acquired at 4 °C predominantly refers to membrane binding of fluorescent compounds. At 37 °C the cells are metabolically fully active, the fluidity of the cell membrane increases and active transport processes can occur. Consequently the cell-associated fluorescence intensity determined at 37 °C refers to binding and uptake. Upon incubation at 4 °C the cell-bound fluorescence intensity remained constant indicating for irreversible binding of the conjugate

to the cell membrane. But after loading comparable amounts of the conjugate at 4 °C, the cell-associated fluorescence intensity decreased upon incubation at 37 °C (Fig. 4). Since fluorescent conjugate dissociated from the loaded cells was not detectable in the supernatant, this decreasing quantum yield might be attributed to uptake of initially membrane-bound conjugate. As the quantum yield of fluorescein is known to be pH-dependent and the fluorescence emission of fluorescein in solution decreases to about 10% at lysosomal pH, transit of the Caco-2 membrane is followed by intracellular accumulation within acidic compartments. The uptake of membrane bound conjugate into the cytoplasm of Caco-2 cells was confirmed by confocal laser scanning microscopy (Fig. 5). As opposed to membrane-binding at 4 °C, at 37 °C binding is followed by uptake of the conjugate into the cells and vesicular accumulation. Thus the lower quantum yield is caused by fluorescein quenching in acidic intracellular compartments. Moreover lysosomal enrichment of F-BSA-WGA might be reconfirmed by the ascent of the 37 °C-curve after 1 h of incubation (Fig. 4). As the conjugate is degraded within the lysosomes, fluorescent low molecular weight degradation products appear in the cytoplasm yielding higher quantum yield due to elevated pH.

The cellular fate of the conjugate and the impact of equilibrating the pH between acidic compartments of the cell and the cytoplasm on cell-associated fluorescence intensity was examined further by treatment of conjugate-loaded Caco-2 cells with monensin (Figs. 6 and 7). Accordingly, by course of time the conjugate–single cell interaction is characterized by four phases: (i) Independent from temperature initially the conjugate is rapidly bound to the cell membrane according to a one way process and not as a result of an equilibrium between binding and dissociation. (ii) Following rapid uptake into the cells, the conjugate is distributed into lysosomal compartments within 1 h. This is confirmed by the decreasing quantum yield at 37 °C as compared with 4 °C and by an only slight increase in quantum yield after equilibrating the intracellular pH. (iii) About 1–4 h after conjugate addition proteolytic degradation of the conjugate within the lysosmes is prevailing. The vast increase of cell-associated fluorescence intensity after addition of monensin points to lower molecular weight degradation products exhibiting a higher quantum yield than the fluorescein-label of the conjugate. Deshielding of intramolecularly quenched fluorescein-label by proteolytic attack was confirmed by incubation of F-BSA with a solution containing trypsin, chymotrypsin and elastase (0.5% w/v each) in HEPES/NaOH buffer pH 8, which resulted in threefold increased fluorescence intensity within 2 h (data not shown). The rather moderate increasing fluorescence intensity in absence of monensin might be attributed to fluorescence quenching of the degradation products due to acidic pH 4.0–5.5 within the lysosomal compartment or cytosolic chymotrypsin- and trypsin-like activities (Bai, 1995). (iiii) Intralysosomal proteolytic breakdown is followed by diffusion into the cytoplasm and excretion of the low molecular, deshielded degradation products of the proteinaceous prodrug. After 5 h the fluorescence intensity of the supernatant strongly increased concurrent with decreasing cell-associated fluorescence intensity in presence of monensin.

Despite the trend of cellular processing of the conjugate in monolayers is similar to single cells, influence of brush border hydrolases on the cellular fate of the conjugate becomes evident upon differentiation of the Caco-2 cells (Howell et al., 1992). Contrary to single cell-experiments, the cell-associated fluorescence intensity initially decreased in absence and presence of monensin (Fig. 8). Consequently after 30 min of conjugate exposure extracellular fluorescent compounds of the conjugate were detected. Thus part of membrane-bound conjugate is degraded by brush border-associated proteases, but the rest is taken up into the cells as indicated by the increasing difference of cell-associated fluorescence intensity in presence and absence of monensin. The conjugate taken up into the cells undergoes a fate similar to that in single cells.

All in all, WGA can mediate cytoadhesion and cytoinvasion of conjugated even high molecular weight proteins to surmount the membrane barrier by temperature dependent active transport mechanisms. Though the lectin resists proteolytic

attack (Gabor et al., 1997), conjugation of protease-labile proteins such as BSA necessitates additional methods of protection. When BSA was transported through Caco-2 monolayers, only 6–9% of the protein in receiver corresponded to completely intact protein as compared with 2% in blood after passage of adult human intestine (Puyol et al., 1995). Thus successful delivery of peptide and protein drugs susceptible to degradation requires formulations ensuring both, protection against proteolytic attack and membrane transition. Conjugation with WGA facilitates cellular uptake, whereas the enzymic barrier might be overcomed by colloidal formulations or enzyme inhibitors.

As wheat germ forms part of the regular diet of man and contains about 300 mg WGA/kg flour, peroral toxicity of the amounts of WGA as necessary for improved intracellular uptake of proteins might be negligible. Though flour is heat treated during baking bread, granola contains a lot of intact WGA, which was not reported to exhibit any negative effects on healthy persons up to now. When rats were fed with 7 g WGA per kg over a 10 days period, less efficient digestion of dietary proteins resulting in reduced growth, increased crypt size and crypt cell proliferation in the small intestine, hypertrophy of the pancreas as well as thymus atrophy was observed (Pusztai et al., 1993). Another obstacle to peroral lectin administration discussed in the literature is modulation of immunogenicity. After oral administration of ovalbumin, in WGA-treated rats no significant increase in anti-ovalbumin IgE was seen as compared with the control lacking WGA administration (Watzl et al., 2001).

Though further studies are necessary, following on from our work WGA-mediated drug delivery offers exciting new perspectives for peroral delivery of proteins to overcome the mucosal barrier.

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