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DISSERTATION

Lectin binding pattern of selected human cell lines and its impact on improved drug delivery

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1 Aims of the present thesis

The surface of mammalian cells is covered by a dense layer of complex carbohydrates. collectively known as the "glycocalyx" or cell coat. These sugar structures represent potential targets for biorecognitive proteins such as lectins, which, by definition, possess at least one non-catalytic domain that binds reversibly to specific mono- or oligosaccharides. Thus, lectins can serve as tools to characterize the glycosylation pattern of cells, which can change during development, differentiation, and malignant transformation. To pursue this major task of the work, various plant lectins with different carbohydrate specificities were applied to the following cell lines: human bladder carcinoma 5637 cells, human chondrocytic C-28/I2 and T/C-28a2 cells as well as primary human chondrocytes, porcine PBMEC/C1-2 and human ECV304 cells mimicking the blood-brain barrier, and human THP-1 cells as model for human monocytes and macrophages. Concerning these cell models, another important aspect of the present work included the establishment of some of the above-mentioned cell lines and a detailed characterization, e.g. via immunofluorescent staining techniques.

After evaluation of the bioadhesive properties of selected lectins and their binding specificity, ongoing focus was set on the mechanisms involved in uptake and intracellular fate of certain lectins being potentially useful as vehicles for lectin-mediated drug delivery. Further studies included the preparation of wheat germ agglutinin (WGA)-functionalized nanoparticles which were applied to untreated and differentiated THP-1 cells.

Based on these studies, an estimation of the carbohydrate composition of the cell lines under investigation should be possible and the potential and feasibility of the lectin-mediated targeting concept determinable.

2 Introduction

The importance of carbohydrates, the most present type of biomolecules in nature and widely expressed as glycoproteins and glycolipids, is well documented for many important biological functions such as cell adhesion, cell-cell recognition, immunological response, inflammation, and proliferation [1-4]. They are major components of the outer surface of all mammalian cells building a dense layer which is called cell coat or glycocalyx. Diversity in glycosylation is found at every level of biological organization, between species, within populations of the same species, and also among different molecules and cell types within the same organism [5]. It has been shown that oligosaccharide structures change during development or differentiation, and abnormal glycosylation is associated with pathological conditions including malignant transformation [6]. Nevertheless, despite numerous efforts, the extent to which the sugar code – a term introduced by H.J. Gabius with a biological information content of 1.44×10^{15} hexasaccharides [7] – has been decoded is still limited. A class of substances, which are capable of recognizing and binding carbohydrates, are lectins of both endogenous and exogenous origin.

Lectins

Definition

Originally identified in plant extracts, lectins are nowadays known to be present in most organisms, ranging from viruses and bacteria to plants and animals [8]. They are considered as a large and heterogenous group of proteins or glycoproteins, which were initially defined as "carbohydrate-binding proteins of non-immune origin which agglutinate cells or precipate glycoconjugates" [9]. That way, some poorly or non-agglutinating proteins were excluded, which were known to contain lectin subunits. Additionally, some lectins possess another binding site that interacts with non-

carbohydrate ligands and thus, lectins were later defined as "carbohydrate-binding proteins other than antibodies and enzymes" [10]. Recently, to be classified as a lectin, a protein or glycoprotein has to meet the following criteria:

1) A lectin is a (glyco)protein that binds carbohydrate.

2) Lectins are separated from immunoglobulins.

3) Lectins do not biochemically modify the carbohydrates which they bind [11].

As the present work concentrates on plant lectins as tools for glycan detection and vehicles for lectin-mediated drug targeting approaches, the definition of Peumans and Van Damme is used in the following papers: Plant lectins can be defined as all plant proteins that possess at least one noncatalytic domain that binds reversibly to a specific mono- or oligosaccharide [12].

Occurrence and distribution

The storage organs of plants are the richest source for plant lectins. In seeds, they typically represent 0.1-5% of the total protein content, but there are examples in some *Phaseolus* species in which lectins account for up to 50% [13]. Lectins were also isolated from roots (e.g., *Phytolacca, Sambucus, Urtica*), tubers or bulbs (e.g., *Allium, Galanthus, Solanum*), bark (e.g., *Maackia, Robinia, Sambucus*), leaves (e.g., *Aloe, Lactuca, Viscum album*), rhizomes, fruits, flowers, ovaries, phloem sap and nectar [11-13]. Lectins, which are found in seeds, are normally confined to them, but there are some legume lectins, which are additionally found in barks. Non-seed lectins can occur in different tissues of the same plant. The potato lectin, for instance, is found in tubers, stems, leaves, and fruits. An exception is the ground elder lectin, which is restricted to the rhizome [13]. The amount of lectin varies markedly among different plants. *Canavalia ensiformis* and *Ricinus communis* contain 2100 and 1400 mg lectin/100 g seeds, respectively, whereas for *Ulex europaeus I* only 16 mg lectin/100 g seeds are found. Other interesting lectins used within this work are *Arachis hypogaea* (190 mg/100 g seeds), *Dolichus biflorus* (110 mg/100 g seeds), *Lens culinaris* (60 mg/100 g seeds), and *Triticum vulgare* (45 mg/100 g seeds) [11].

Carbohydrate specificity

According to the ability of lectins to bind one or more monosaccharides, lectins were classified into five groups: mannose/glucose-, *N*-acetylglucosamine-, *N*-acetylgalactosamine/galactosamine-, fucose-, and sialic acid-binding lectins [14]. Except for fucose, sugars are of D configuration. Other specificities are found rarely, as these sugars are also typical components of the surfaces of eurkaryotic cells. As an exception, the human serum amyloid P component, a lectin occuring in certain algal polysaccharides, in a marine sponge, and in a yeast, binds to 4,6-cyclic pyruvate acetal of galactose [8]. Lectins, which bind solely oligosaccharides are excluded from this classification (e.g., *Griffonia simplicifolia IV*, *Phaseolus vulgaris*). Thus, they are mostly regarded as lectins with a "complex specificity". Interestingly, many plant lectins, which are able to bind monosaccharides, have a higher affinity for oligosaccharides, which are not common or even absent in plants [12]. For instance, lectins from elderberry (*Sambucus* sp.) and *Maackia amurensis* bind to a major carbohydrate constituent of animal glycoproteins absent in plants [12]. As an important consequence of the preference of plant lectins for sugar components from animal or microbial origin, most lectins seem to be aimed at foreign glycoconjugates rather than endogenous receptors Thus, plant lectins are regarded as useful tools for the isolation and analysis of human and animal glycoconjugates with impact on clinical applications [13].

Lectin-carbohydrate interaction:

Comparable to other carbohydrate-binding proteins, lectins combine with carbohydrates primarily via a network of hydrogen bonds and hydrophobic interactions. In some cases, electrostatic interactions and coordination with metal ions are also involved [8]. Hydrogen bonds are formed between the hydroxyl groups of the carbohydrate and the amino side chains of the lectin, frequently including aspartic acid, glutamic acid, glutamine and araginine residues. Occasionally also hydroxyl side chains of the protein are involved [15].

Moreover, van der Waals interactions being commonly present may contribute significantly to binding, though usually characterized as rather weak forces. The steric disposition of the hydroxyl groups of carbohydrates normally representing hydrophilic molecules can result in creation of hydrophobic patches on the sugar surfaces which then forms contacts with hydrophobic side chains of the protein [8]. For example, a methyl group of *N*-acetylamino sugars may interact with aromatic residues in lectins [8].
Ionic interactions occur rarely as they require charged molecules, and saccharides are mostly uncharged. An exception is the bond between a histidine of the cation-dependent Man6*P* receptor and the phosphate of Man6*P* [15].
Water has the ability to serve as hydrogen donor as well as acceptor. That way water can mediate a lectin-carbohydrate interaction by forming bridges, which commonly consist of a single water molecule [15].

Due to the binding capacity of lectins to carbohydrates of the cell surface, they might be used as carrier molecules to target drugs specifically to particular cells and tissues. In this context, two approaches are followed to date: the prodrug-concept as represented by soluble lectin-drug conjugates and surface modified carrier systems [16]. For both drug delivery systems, besides cytoadhesion, cytoinvasion of the lectin is desirable. Uptake of different lectins has been observed in various studies, for instance for tomato lectin found to be transported across the intestinal epithelium [17, 18], for peanut agglutinin located in peripheral venous blood after oral administration [19]. Transcytosis of *Ulex europaeus* agglutinin-I was assessed after binding to mouse M-cells [20]. Thus, complementing the evaluation of lectins for drug delivery purposes, the cytoinvasive potential of selected lectins was assessed during this thesis.

Application and function of lectins:

Some fields of application for lectins were already mentioned above. A recent summary was given by Gabius et al [21]:

Table I: Plant lectins as research tool, taken from [21]

Biochemistry
detection of defined carbohydrate epitopes of glycoconjugates in blots or on thin-layer chromatography plates purification of lectin-reactive glycoconjugates by affinity chromatography glycan characterization by serial lectin affinity chromatography (lectin affinity capture) glycome analysis (glycomics) quantification of lectin-reactive glycoconjugates in enzyme-linked lectin-binding assays (ELLA) quantification of activities of glycosyltransferases/glycosidases by lectin-based detection of products of enzymatic reaction model reagents for the assessment of the ligand functionality of carbohydrate-presenting scaffolds (e.g. glycodendrimers)
Cell biology
characterization of intracellular assembly, routing, and cell surface presentation of glycoconjugates in normal and genetically engineered cells (glycomic profiling, spatially defined) selection of cell variants (mutants, transfectants) with altered lectin-binding properties as models for dissecting glycosylation machinery and glycan functionality (glycomic profiling, functionally defined) fractionation of cell populations modulation of the proliferation and activation status of cells and dissection of the involved signal pathways model substratum for study of cell aggregation, adhesion, and migration
Medicine
detection of disease-related alterations of glycan synthesis by lectin cyto- and histochemistry histo-blood group typing and definition of secretor status quantification of aberrations of cell surface glycan presentation, e.g. in malignancy cell marker for diagnostic purposes including marking infectious agents (viruses, bacteria, fungi, parasites) cell marker for functional assays to pinpoint defects in cell activities such as mediator release

Despite the various options for the use of lectins, their physiological role is often discussed controversially. Again, Gabius et al have recently compiled a summary as shown in Table II.

Table II: Physiological role of plant lectins, taken from [21]

	Activity	Example of lectin
external activities	protection from fungal attack	*Hevea brasiliensis* (rubber tree), *Urtica dioica* (stinging nettle), *Solanum tuberosum* (potato)
	protection from herbivorous animals	*Phaseolus vulgaris* (French bean), *Ricinus communis* (castor bean), *Galanthus nivalis* (snowdrop), *Triticum vulgare* (wheat)
	involvement in establishing symbiosis between plants and bacteria	*Pisum sativum* (common pea), *Lotononis bainesii* (miles lotononis), *Arachis hypogaea* (peanut), *Triticum vulgare* (wheat), *Oryza sativa* (rice)
Internal activities	storage proteins	valid for all lectins
	ordered deposition of storage proteins and enzymes in protein bodies and mediation of contact between storage proteins and protein body membranes	*Pisum sativum* (common pea), *Lens culinaris* (lentil), *Glycine max* (soybean), *Oryza sativa* (rice)
	modulation of enzymatic activities such as phosphatase activity	*Secale cereale* (rye), *Solanum tuberosum* (potato), *Pleurotus ostreatus* (oyster mushroom), *Glycine max* (soybean), *Dolichos biflorus* (horse gram)
	participation in growth regulation	*Medicago sativa* (alfalfa), *Cicer arietinum* (chick pea)
	adjustment to altered environmental conditions	*Triticum aestivum* (winter wheat)

Lectins used in the following studies

To cover a broad range of possible carbohydrate structures at the cell surface, lectins with distinct specificities were chosen as follows:

Wheat germ agglutinin (WGA), *Solanum tuberosum* lectin (STL), *Lens culinaris* agglutinin (LCA), *Ulex europaeus* isoagglutinin I (UEA-I), *Sambucus nigra* agglutinin (SNA), *Dolichus biflorus* agglutinin (DBA), and peanut agglutinin (PNA).

Each lectin is characterized in detail in Table III.

Table III: Plant lectins used in the following studies.

Lectin	MW	Carbohydrate specificity	Structure
WGA	36 000	GlcNAc, sialic acid	dimeric carbohydrate free protein of 2 identical subunits, which contain four repetitive domains; mixture of 3 isoforms
STL	100 000	GlcNAc	dimeric protein of 2 identical subunits, which contain up to 40-50% covalently linked carbohydrate;
PNA	110 000	ß-D-Gal-D-GalNAc, ß-D-GalNAc, Gal	homotetrameric non-glycosylated protein of 4 subunits; complex mixture of isolectins
DBA	120 000	α-D-GalNAc, Gal	heterotetrameric glycoprotein

UEA	63 000	α-L-Fuc	dimeric glycoprotein of 2 different subunits; isolectin I
SNA	150 000	Sialic acid α(2,6)Gal/GalNAc	glycoprotein of 4 units [A-s-s-B]; isolectin I
LCA	49 000	α-Man, α-Glc, α-GlcNAc	protein composed of 2 α-chains and 2 ß-chains; mixture of 2 isoforms

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

Cell cultures

As already mentioned above, the present work focuses on the application of lectins to characterize the glycosylation pattern of different cell types, in order to compare the glycosylation profiles, to detect alterations in the glycocalyx during differentiation of cells, to follow cytoinvasion of selected lectins, and to provide thereby a suitable basis for lectin-mediated drug delivery approaches. To accomplish these goals, the following cell lines were used during this work:

5637 cells as model for human bladder carcinoma,

C-28/I2, T/C-28a2, and primary human chondrocytes for cartilage research,

PBMEC/C1-2 and ECV 304 cells for characterization of the blood-brain barrier, and

THP-1 cells as model for human monocytes which undergo differentiation to macrophages.

In general, cell cultures play an important role in the first stages of pharmaceutical development. They can be applied for numerous purposes including studies on cell physiology, metabolism, protein expression, and they represent tools for analysis of active and passive transport of drugs, for toxicity tests, and for the development of specific delivery systems. In contrast to animal models, which have been in use for a long time to evaluate biopharmaceutical characteristics of new pharmaceutical ingredients but with clear limitations due to divergent species specificities, the complex nature of the

model as well as ethical reasons, cell culture models offer the advantage of being a defined system in which parameters and conditions can be changed as required. Nevertheless, for a precise handling and accurate interpretation of results, a detailed characterization and reliable standardization is essential. In this context, another important aspect of the present work was set on the establishment of some of the above-mentioned cell lines including a careful characterization using for example immunofluorescent staining techniques.

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3 Specific topics

Author´s contribution

I hereby declare to have significantly contributed to the realization of the studies included in the present thesis.

Regarding the **first part** (Targeted drug delivery: Binding and uptake of plant lectins using human 5637 bladder cancer cells) I contributed to the study design, carried out the cell culture, and performed some experiments. Moreover, I interpreted the results and wrote the manuscript. The review (Strategies to improve drug delivery in bladder cancer therapy) was prepared together with the other authors.

In the **second part** of the thesis (papers: Lectin binding studies on C-28/I2 and T/C-28a2 chondrocytes provide a basis for new tissue engineering and drug delivery perspectives in cartilage research and Lectin binding patterns reflect the phenotypic status of in vitro chondrocyte models) I participated in conceiving the studies, contributed to the maintenance of the cell culture and chondrocyte isolation, performed the lectin-cell interaction experiments and the immunofluorescent staining of the cells, and was involved in data analysis and interpretation.

In the **third part** (manuscripts: Characterization of two blood-brain barrier mimicking cell lines: distribution of lectin binding sites and perspectives for drug delivery and Alteration of the glycocalyx of two blood-brain barrier mimicking cell lines is inducible by glioma conditioned media) I contributed to the study design, performed the lectin-interaction experiments, was involved in data analysis and interpretation, and I wrote one of the manuscripts.

In the **fourth part** (Alteration of the glycosylation pattern of monocytic THP-1 cells upon differentiation and its impact on lectin targeting) I contributed to the study design, I established the cell culture, performed some of the lectin-interaction studies, was involved in data analysis and interpretation, and I wrote the manuscript.

Vienna, April 2009

Part I: bladder

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Part I: bladder

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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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Part I: bladder

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

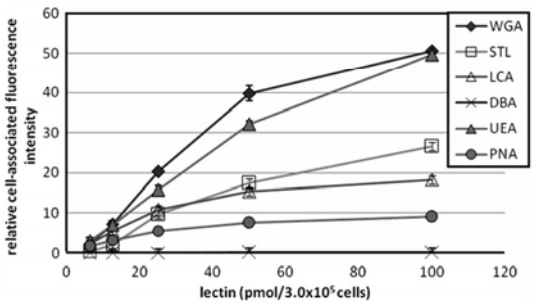

Fig. 1. Lectin binding to 5637 single cell at 4 °C. The fluorescein-labelled lectins associated with the cell surface were related to an apparent F/P ratio of 1 (mean ± SD, $n = 3$).

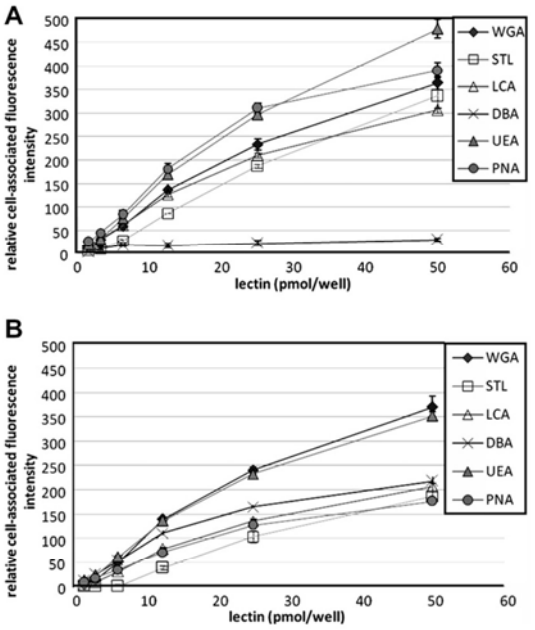

Fig. 2. Lectin binding to 5637 cell monolayers cultivated for 4 d (A) or 11 d (B) at 4 °C. The fluorescein-labelled lectins associated with the cell surface were related to an apparent F/P ratio of 1 (mean ± SD, $n = 4$).

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%.

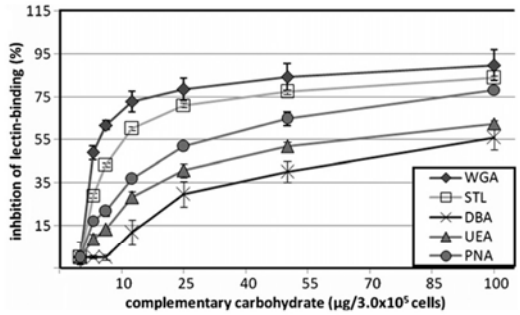

Fig. 3. Competitive inhibition of lectin binding to 5637 single cells by addition of increasing amounts of complementary carbohydrate (mean ± SD, $n = 3$).

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-

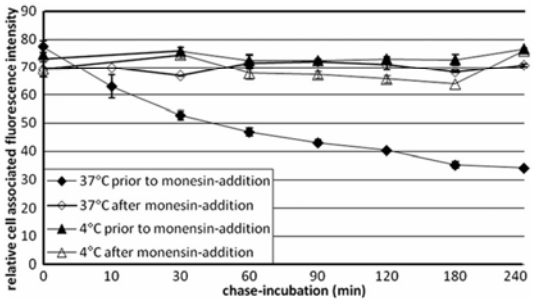

Fig. 4. Relative cell-associated fluorescence intensities of WGA-loaded 5637 single cells prior and after monensin addition during incubation at both 4 °C and 37 °C by time up to 4 h (mean ± SD, $n = 3$).

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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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Strategies to Improve Drug Delivery in Bladder Cancer Therapy

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Part I: bladder

Abstract

Bladder cancer is the ninth most common malignancy in the world featuring a very high gender variability in occurrence. Current options for bladder cancer therapy include surgery, immunotherapy, chemotherapy, and radiotherapy with a trend towards multimodal treatments. However, successful management remains a challenge for urologists and oncologists due to the high risk for recurrence and progression. Especially in the field of bladder cancer chemotherapy, efficacy of treatment might be improved by advanced drug delivery strategies aiming at prolonged residence time within the bladder cavity and increased permeability of the bladder wall during intravesical instillation. Moreover, a deeper understanding of the biology of bladder carcinogenesis and progression stimulated the development of a new generation of anti-cancer drugs for targeted therapies which might result in an increased treatment specificity together with a lower toxic potential and higher therapeutic indices. The present review will discuss the currently available strategies for "targeted therapy" focusing on molecular targets and for "controlled delivery" comprising all other approaches towards improved drug delivery.

Keywords: bladder cancer; chemotherapy; controlled delivery; drug targeting; intravesical delivery; targeted therapy; urothelial cell carcinoma

1. Introduction

Recent literature ranks bladder cancer as the ninth most common malignancy worldwide together with a very high gender variability in occurrence [1]. Interestingly the incidence of bladder cancer varies significantly among countries with highest rates in the Western world. Particularly high incidence rates were observed for Southern Europe, Northern Africa, North America and Western Europe; occurrence in Asian countries is rather low [2]. In the USA, bladder cancer is the fourth most common cancer diagnosed in males with estimated 51,230 new cases and 9,950 deaths from bladder cancer in 2008 [3] The two most well established risk factors for bladder tumour include cigarette smoking and occupational exposures to urothelial carcinogens. In addition, other environmental exposures like chronic urinary tract infections, cyclophosphamide use, schistosomiasis, and exposure to radiotherapy as well as inadequate consumption of fruits, vegetables, and certain vitamins may be associated with risk of bladder cancer. Even coffee consumption and artificial sweeteners are discussed to have an impact on tumorigenesis, while familial bladder cancer is fairly rare as compared to other tumour sites [4].

In general, bladder cancer is a rather heterogeneous disease making classification, staging and grading a challenging task. On average, 70% of bladder urothelial cell carcinomas represent a superficial disease and the remainder develops a muscle-invasive disease bearing the risk of metastatic spread of the tumour [5]. Standard treatment for superficial, non muscle-invasive bladder cancer is complete transurethral resection and subsequent cystoscopic surveillance. However, the major challenge in the management of superficial bladder cancer is not the removal of a lesion once present but rather the prevention of tumour recurrence and progression. Thus, intravesical instillation of chemotherapeutic or immunotherapeutic agents is applied to reduce the risk for recurrence and to delay or even prevent progression to a muscle-invasive disease. Independent of the modality of treatment, two-thirds of patients diagnosed with superficial bladder cancer suffer from a recurrence of their tumour and 10–20% of those that recur demonstrate progression to muscle-invasive disease [6]. A tumour that has grown beyond the lamina propria and invades the muscularis propria is usually treated with radical cystectomy. Radical surgery

in the case of muscle-invasive bladder cancer therapy is usually accompanied by perioperative systemic chemotherapy to minimize metastatic dissemination and to improve survival of patients. For patients with disseminated disease aggressive systemic therapy with multiple chemotherapeutics is essential [7].

Especially in the field of cancer chemotherapy the urgent need for improved drug delivery by directing the active pharmaceutical ingredient (API) to the diseased tissue stimulates the research efforts of pharmaceutical technologists since more than one decade. That way, due to their severe side-effects, the dose-limited effectiveness of conventional anti-cancer drugs might be overcome [8]. Improving drug delivery by guiding the drug to the desirable site of action is also a relevant issue in anti-cancer therapy of the bladder. Due to its unique anatomical and physiological properties the urinary bladder is ideally suited for minimally invasive intravesical therapy, an option that is widely used in the treatment of urothelial cancer at the superficial stage. However, despite the fairly easy access by instillation, intravesical delivery of effective concentrations still remains a challenge. The primary goal in intravesical drug administration is to maximize the exposure of the tumour to the therapeutic agent while limiting toxicity to the host. Due to the barrier function of the urothelium absorption of the API into the systemic circulation is limited avoiding overall toxicity, but the low residence time of a drug in the bladder, which rarely lasts beyond the first void, requires advanced and intelligent approaches towards sustained delivery in order to achieve efficacious drug concentrations within the bladder cavity. Representing one of the toughest barriers in the human organism [9], the transport across the urothelium occurs only via passive diffusion. Thus, improving the permeability of the bladder epithelium as well as increasing the concentration gradient between the urine and the target site is expected to enhance drug penetration into the diseased tissue. Besides some more general physiological approaches, interesting methods in bladder cancer therapy comprise chemical agents that alter the urothelial permeability as well as device-assisted therapies, such as electromotive drug administration or thermo-chemotherapy. A prolonged residence time of the API in the bladder is achieved via several sustained drug delivery concepts including thermosensitive hydrogel formation *in situ*, mucoadhesion,

magnetically targeted carriers, and drug carriers from the nanotech pipeline such as liposomes, micro- or nanoparticles. Moreover, photodynamic therapy is employed in bladder cancer treatment.

Recent knowledge of the molecular mechanisms underlying bladder carcinogenesis as well as tumour progression and response led to a further approach in bladder cancer therapy that is fundamentally different to the others mentioned and relies on molecular targeting of tumour relevant signalling pathways, cell cycle regulators, and specific receptors that are overexpressed at the tumour cell surface. Promising molecular targets in bladder cancer therapy include the epithelial growth factor receptor family, the Ras and phosphatidylinositol-3-kinase signalling pathway, cyclin-dependent kinases, and several others. The expected benefits of molecularly targeted therapies rely on a lower overall toxicity due to an increase in selectivity of the therapeutic agent. Moreover, the greater specificity might also show promise for successful therapy of advanced or metastatic disease. Further concepts in targeted bladder cancer treatment are based on gene therapy. Amongst others, potential targets in this area are the tumour suppressor gene p53, telomerase reverse transcriptase, and the apoptosis modulator bcl-2. Besides, an interesting focus of recent research deals with the prevention of metastatic spreading by the inhibition of neo-angiogenesis particularly in the management of advanced bladder cancer. In addition, glycotargeting via lectins might prove to be a promising alternative approach to selectively target malignant cells of the bladder.

Below, the term “targeted” will be used primarily for molecular targeting, while “controlled” will be used for all others methods of improved drug delivery.

2. Controlled delivery

Bladder tumours at a superficial stage are currently treated by transurethral resection followed by adjuvant instillation of chemotherapeutics or immunotherapeutics to lower the risk of recurrence and progression to muscle-invasive disease. For chemotherapy usually mitomycin C (MMC) and epirubicin are applied either as a single immediate instillation or multiple delayed instillations depending on the patient´s risk evaluation. Intravesical immunotherapy is predominantly done with bacillus Calmette-Guérin (BCG), an attenuated mycobacterium known as a potent inducer of passive immunity which is the best choice for high-risk patients even though local and systemic side-effects are more frequent and more severe than with intravesical chemotherapy [10]. Gold-standard therapy in case of muscle-invasive bladder cancer relies on radical cystectomy usually in conjunction with perioperative systemic chemotherapy using a combination of methotrexate, vinblastine, doxorubicin and cisplatin (M-VAC) which proved to be substantially more effective than any single drug regimen [11,12]. M-VAC therapy is also a typical regimen for the treatment of metastatic carcinoma of the urothelium [13]. Moreover, a multi-drug combination chemotherapy using cisplatin, methotrexate, vinblastine (CMV) is applied in case of metastatic bladder cancer [14].

Besides approaches relying on new chemotherapeutic agents and regimens [15] improvements in drug delivery have been in the focus of recent research in order to enhance the efficacy of bladder cancer therapy. Among the variety of efforts in this field, which will be discussed in detail below, there are also some general aspects in terms of the physiological status and function of the kidney and the bladder. These parameters include urine factors such as the residual volume in the bladder and the continuous urine production rate during intravesical treatment both resulting in dilution of the drug administered within the instillation fluid. A clever strategy to circumvent these problems is based on complete emptying of the bladder prior to dose administration as well as limited fluid intake by the patient prior and after instillation. In addition, the dosing volume and the dwell time are perceptible parameters, but their impact is lower [16]. Moreover, pH might be an issue depending of the chemotherapeutic agent in use. For

MMC rapid degradation in acidic environment could be avoided by alkalinizing the urine by oral administration of sodium bicarbonate. Together with an increased dosage of the cytostatic agent, a decrease of the instillation volume and considering the above mentioned parameters the efficacy of MMC treatment could be statistically significantly enhanced in a randomised phase III trial [17]. Another crucial point in intravesical chemotherapy is the vehicle used for drug administration. According to Groos et al. [18] sterile water might be a better diluent than saline due to the fact that osmolality was inversely correlated to the toxicity of chemotherapeutical agents in cell cultures. However, the residence time of the API at the site of action is a further decisive parameter often limiting efficient cancer treatment. A simple and reasonable way towards increased drug delivery to the bladder epithelium is prolonged instillation, but also more advanced approaches have already been pursued as follows.

2.1. Thermosensitive Hydrogels

To extend the residence time of the API in the bladder beyond the first voiding of urine postinstillation, the application of a thermosensitive hydrogel forming a drug depot inside the baldder cavity was proposed by Tyagi et al. [19]. Developed by Jeong et al. [20], the aqueous solution of the triblock co-polmyer *PEG-PLGA-PEG* (Polyethylene glycol-poly[lactic acid co-glycolic acid]-polyethylene glycol) is fluid at room temperature and converts to a gel at body temperature. Using rat bladders and a modified thermogel, Tyagi et al. [19] reported a sustained release up to 24 h provided by the thermosensitive hydrogel which attaches itself as a smooth layer on the inner surface of the bladder and acts as a drug-loaded matrix. Although representing a promising approach, human data are not available yet.

2.2 Mucoadhesion

Another approach to enhance drug transport is the concept of bioadhesion to the mucosal urothelium. This is the process whereby natural or synthetic macromolecules adhere to mucosal surfaces in the body and, when incorporated into pharmaceutical formulations, absorption of drugs can be enhanced due to prolonged exposure, shortening of the diffusional pathway and increasing the concentration gradient from the lumen to the cell. For *chitosan* and *polycarbophil*, permeation enhancement across the bladder wall has been assessed in previous studies on porcine bladders [21,22]. For specific post-operative chemotherapy, MMC loaded *chitosan* and *alginate* carriers were prepared by Oztürk et al. [23] which proved successful in mice. Lee et al. [24] investigated the effect of formulations with paclitaxel and *glyceryl monooleate* in a rabbit model of bladder cancer. They observed an increased bioadhesiveness to bladder mucosa providing a step forward towards targeting the bladder tissue. Ongoing studies focus on increased efficiency of drug absorption by API-incorporation into liposomes, micro- and nanoparticles with bioadhesive characteristics as discussed below.

2.3. Drug carrier systems

Liposomes

Liposomes are artificial vesicles of spherical shape containing an aqueous core surrounded by one or more phospholipid layers. Thus, liposomes can be used as carrier systems for either hydrophilic or lipophilic compounds and can therefore alter pharmacologic and pharmacokinetic parameters of the transported drug. In a fundamental study Johnson et al. [25] determined whether liposomes can bind to human bladder cancer cells. Binding to tumor cells was saturable and appeared to be specific as binding of liposomes to normal fetal bladder cells was negligible. Thus, they might be a vehicle to improve the delivery of anticancer drugs. Vail et al. [26] encapsulated doxorubicin in polyethylene glycol-coated liposomes and showed in animal models an increased

potency over conventional doxorubicin as well as remission and cure of many cancers including the bladder. Anti-tumor effects of liposome-encapsulated titanium dioxide in bladder cancer treatment were examined by Chihara et al [27] in nude mice and revealed higher anti-tumour effects than non-coated titanium oxide. A recent study was performed by Humhuan et al. [28] who incorporated the BCG cell wall into cationic liposomes and demonstrated their uptake into murine bladder tumor cells.

Micro- and Nanoparticles

Polymeric micro- and nanoparticles have become widely applied as effective drug delivery systems. To prolong the residence time of paclitaxel in the bladder and to achieve controlled release, bio-adhesive *poly(methylidene malonate-2.1.2) microspheres* were prepared by Le Visage et al. [29], which were applied to Balb/c mice after *N*-butyl-N(4-hydroxybutyl)nitrosamine (BBN)-induction of bladder cancer and compared to paclitaxel. After bladder instillation, microspheres adhered to the mucosa, remained in the bladder for up to 48 h and the 9-week survival rate was significantly improved. Lu et al. [30] developed paclitaxel-loaded *gelatin-nanoparticles* that rapidly released paclitaxel, yielded significant activity against human bladder cancer cells and showed higher tissue concentration as compared to commercial Cremophor®/EtOH formulations. Paclitaxel was also loaded in *PLGA nanospheres* to increase both efficiency and safety of the drug as well as to improve targeting [31]: Tested on thyroid, breast and bladder cancer cell lines these nanospheres showed a prolonged drug release and an increase of the cytotoxic effect with respect to free paclitaxel in all cell lines. Recently, paclitaxel was incorporated in *nanoparticles* made from hydrophobically derivatized *hyperbranched polyglycerols*. Their evaluation in nude mice with orthotopic KU7-luc tumors [32] revealed that HPG-C10-polyethylenglycol of paclitaxel was significantly more effective in reduction of tumour growth than paclitaxel formulated in Cremophor®. Another recently published study concentrates on the delivery of MMC encapsulated in cationic *nanoparticles of chitosan and polycaprolactone* [33]. Nanoparticles of poly-ε-caprolactone coated with chitosan turned out to be an efficient formulation for a complete drug release and specific uptake by MB49 bladder carcinoma cells in contrast to normal

bladder cells. Recently, *carbon nanotubes* (CNTs) turned out to be a feasible carrier for carboplatin [34] as cell growth of human bladder cancer cells was inhibited in comparison to unfilled CNTs.

Anchoring of antibodies, lectins, etc. on the surface of liposomes or micro- and nanospheres can further improve the site specificity of drug carriers and will be discussed in detail in section 3.

2.4. Permeation enhancement: Chemical Approaches

Dimethyl sulfoxide (DMSO) is a chemical compound with the distinct capability to penetrate tissues. Co-administration of *DMSO* proved to enhance the penetration of cisplatin in the urinary bladder of dogs [35], and improved the absorption of doxorubicin [36], pirarubicin [37], and epirubicin [38] in rat models. *DMSO* also reversed the entrapment of paclitaxel in Cremophor micelles, which is a commercial available formulation (i.e., Taxol®). Summarising all the effects, co-administration with *DMSO* resulted in increased delivery of paclitaxel to bladder tissue of dogs [39].

Sasaki et al. [40] evaluated the effects of a certain *saponin* on absorption of 4`-O-tetrahydropyranyldoxorubicin (THP) through the bladder mucosa of rats. As the results revealed increased concentrations of THP in bladder tissue without affecting that in plasma, co-administration could be useful for intravesical chemotherapy.

Hyaluronidase, an enzyme that degrades hyaluronic acid representing a major constituent of the extracellular matrix, had been administered to improve drug diffusion of cisplatin in 33 patients with superficial transitional bladder cell carcinoma [41]. Higher clinical efficacy over cisplatin alone was not detected, but a further study revealed a decrease in tumor recurrence after co-administration of MMC with *hyaluronidase* [42] in a randomized trial with 2 groups of 28 patients with highest significance ($p < 0.05$). This finding was verified in comparative studies with 43 patients treated with MMC and *hyaluronidase* (recurrence rate 7%) and 63 patients with MMC alone (recurrence rate

32%) [43]. Nevertheless, the feasibility of this concept has to be carefully evaluated since *hyaluronidase* can either act as tumor suppressor or promoter depending on cell type and concentration [44,45].

2.5. Permeation enhancement: Physical Approaches - Device-assisted therapy

Electromotive drug administration (EMDA)

The technique of electromotive drug administration is known to increase penetration of drugs through the urothelial barrier of the bladder after intravesical instillation by the use of an electric source. Based on the active principles of iontophoresis, electroosmosis and electroporation the transport of water-soluble drugs is enhanced and is most effective for ionized molecules where the rate of drug transport is proportional to the intensity of the applied current [46,47].

In the treatment of bladder cancer a recent study was conducted by Colombo et al. [48] with patients suffering from single, recurrent, low stage, low grade superficial bladder tumour. 36 patients were treated with the standard procedure administering MMC as intravesical instillation, for 15 patients MMC was applied according to the electromotive procedure and in 29 patients MMC was administered in combination with local microwave-induced hyperthermia. Complete response was identified in 27.7%, 40% and 66% of patients, respectively. For high-risk superficial bladder cancer Di Stasi et al. [49] assessed the efficacy of intravesical electromotive versus passive MMC using BCG as a comparative treatment in 108 patients. After 6 months follow-up, the complete response for electromotive vs passive MMC was 56% vs 31%, for BCG 64%. Local and systemic side effects were significantly more frequent in the BCG arm, leading to the suggestion that electromotive MMC may be an alternative therapy. In a randomised trial with 212 patients with stage pT1 bladder cancer, the same authors compared the efficacy of sequential BCG and electromotive MMC with that of BCG alone [50]. After a median follow-up of 88 months, more patients assigned sequential BCG and electromotive MMC were disease-free (57.9 % vs 41.9%), higher disease-free intervals were achieved (69

months vs 21 months), progression rate was lower (21.9% vs 9.3%) and the overall (34% vs 21.5%) as well as disease-specific mortality (16.2% vs 5.6%)) was lower as compared to BCG alone.

Hyperthermia

Recently, intravesical hyperthermia was applied to improve the efficacy of chemotherapeutics termed as thermo-chemotherapy. Colombo et al. [51] developed the Synergo® system (Medical Enterprises Ltd, Amsterdam, The Netherlands) which consists of a 915 MHz intravesical microwave applicator, inserted through a catheter, that provokes hyperthermia of the bladder wall via direct irradiation. The bladder wall temperature is monitored by thermocouples located inside the catheter (goal temperature 42-43°C) and a small peristaltic pump circulates the chemotherapeutic solution between the bladder and a drug reservoir. The efficacy of hyperthermia in combination with MMC versus MMC alone was evaluated in several clinical studies [52-54] and revealed to be more effective than intravesical chemotherapy alone for the treatment of superficial transitional cell carcinoma. A comprehensive study with 90 patients suffering from intermediate or high risk superficial transitional cell carcinoma was performed by Van der Heijden et al. [55]. After 2 years of follow-up the risk of recurrence was 24.6 % and no progression in stage and grade was observed. A current study from Witjes et al. [56] evaluated the potential of thermo-chemotherapy in 57 patients with mostly BCG refractory carcinoma in situ. A complete response rate of 94% was achieved with a recurrence rate of 30% at 1 year follow-up.

2.6. Photodynamic therapy

Photodynamic therapy (PDT) is a potentially selective approach for treating bladder cancer based on the interaction between a photosensitising agent in the target tissue and an intravesical light source to cause tissue necrosis. Using 5-aminolevulinic acid (5-ALA) as a precursor of the potent photosensitiser protoporphyrin IX, a first clinical

experience for treatment of superficial bladder cancer was achieved by Kriegmair et al. [57]. After 10-12 weeks, 4 out of 10 patients had a complete and 2 partial remission, no change was detected for 3 patients and 1 suffered from progressive disease. Additionally, no serious side-effects were assessed. The same authors evaluated the efficacy of photodynamic therapy after oral administration of 5-ALA in 24 patients with superficial bladder cancer [58]. At a median follow-up of 36 months, 3 of 5 patients with carcinoma in situ and 4 of 19 patients with papillary tumours were free of recurrence. 3 patients were rendered disease-free by repeated therapy and 3 underwent cystectomy. But hemodynamic side effects such as hypotension and tachycardia, especially in patients with cardiovascular co-morbidity, must be taken into account. Thus, efficacy and side effects of PDT with intravesical application of 5-ALA were evaluated again by Berger et al. [59] with 31 patients suffering from recurrent superficial bladder cancer. 16 patients were recurrence free after an average follow-up of 23.7 months and therapy was well tolerated. The only side effects observed were dysuria and haematuria in 4 and 7 patients, respectively. Evidence for increased susceptibility of bladder cancer cells to chemotherapeutic agents after sequential administration of MMC and PDT was given by French et al. [60] in a preclinical study. In an ongoing phase-1 study with 24 patients with recurrent superficial bladder cancer, Skyrme et al. [61] assessed the potential and tolerability of ALA-PDT in association with MMC and found out that this procedure is safe, well-tolerated and able to manage difficult-to-control superficial transitional cell carcinoma and carcinoma *in situ* of the bladder. Improvements are in progress, on the one hand concentrating on newer generations of photosensitisers such as hypericin [62,63], on the other hand on novel targets such as the transferrin receptor for more specific therapy [64] or p38α MAPK inhibition to avoid tumour survival and recurrence [65].

3. Targeted therapy

Whereas controlled delivery focuses on improving the overall drug exposure to the bladder, targeted therapies are selectively directed to cancer cells or the processes involved in their genesis and metastasis. Tumorigenesis is a multistep process that transforms normal human cells into malignant derivates. Among these steps, self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, unlimited replicative potential, sustained neo-angiogenesis, and tissue invasion and metastasis are regarded as essential alterations towards malignant growth [66]. The improved understanding of the biology of bladder carcinogenesis and tumor progression has led to the identification of specific genetic lesions as well as biochemical receptors and signal transduction pathways which should improve cancer diagnostics and can also serve as novel therapeutic targets.

3.1. Epidermal growth factor receptor (EGFR) family

The epidermal growth factor receptor family comprises four homologue receptors: ErbB1 (EGFR, HER1), ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4 (HER4). They are composed of an extracellular binding domain, a transmembrane lipophilic segment, and an intracellular tyrosine kinase domain. Activation plays a crucial role in many cell regulatory processes such as proliferation, migration, and modulation of apoptosis leading to the progression of many malignancies including bladder cancer [67,68].

Epidermal growth factor receptor (HER-1)

The epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane protein, which is normally expressed only on the basal layer of bladder epithelial cells. Upon malignant transformation EGFR is also highly expressed in the superficial layers of tumors and overexpression is detected in 31-48% of bladder cancer associated with progression of the tumor and a poor prognosis [69-72]. Being identified as a potential

molecular target, two major approaches are followed: monoclonal antibodies directed at the extracellular domain of the receptor and inhibition of the receptor´s tyrosine kinase.

Regarding *monoclonal antibody therapy*, a promising candidate is IMC-C225/cetuximab (Erbitux®), a human/murine chimeric monoclonal antibody against EGFR. Perrotte et al. [73] showed in nude mice with metastatic human transitional bladder cancer that therapy with cetuximab had a significant antitumor effect, which was partially mediated by inhibition of angiogenesis. A study conducted by the same authors [74] further investigated whether the combination with paclitaxel could enhance this therapeutic effect. Again, this treatment down-regulated the expression of basic fibroblast growth factor, vascular endothelial cell growth factor, interleukin-8, and matrix metalloproteinase type 9 and inhibited tumor-induced neovascularisation compared with untreated control ($p<0.005$). This combination also enhanced apoptosis in tumour and endothelial cells as compared with drug alone ($p<0.005$). Current data of clinical studies with cetuximab in combination with chemotherapy are available for treatment of head and neck cancer [75], non-small cell lung cancer [76], and colorectal carcinoma [77]. For bladder cancer a phase II randomized trial evaluating the effects of gemcitabine and cisplatin with or without cetuximab is currently recruiting participants [78].

ZD 1839/gefitinib (Iressa®) is an orally active EGFR *tyrosine kinase inhibitor* (TKI) that interacts with the intracellular tyrosine kinase domain and subsequently inhibits downstream signalling [79]. A basis for the application in bladder cancer treatment was provided from Nutt [80] and Dominguez-Escrig [81] who studied the therapeutic potential in preclinical bladder cancer models and evaluated the therapeutic value. In a phase II clinical trial, gefitinib has been tested in combination with cisplatin and gemcitabine in 27 untreated advanced transitional cell carcinoma patients [82]. In summary, the trial showed activity in advanced transitional cell carcinoma, but the relative contribution of gefitinib was not assignable and this regimen was associated with excessive toxicity. Currently, a randomised phase II study is being conducted for further evaluation [78].

In contrast to gefitinib, where the U.S. Food and Drug Administration (FDA) has restricted the use in advanced non-small cell lung cancer to patients participating in a clinical trial or continuing to benefit from treatment already initiated, OSI-774/erlotinib (Tarceva®), another small molecule TKI, is approved by the FDA for therapy of advanced non-small cell lung cancer and is continued to be investigated [83]. Associating this concept with bladder cancer, Yang et al. [84] assessed the effects of erlotinib in comparison to interferon-α on bladder cancer cell lines. Both, erlotinib and interferon-α were significantly antiproliferative, and combined treatment enhanced the sensitivity of most cell lines. Another study evaluated the epidermal growth factor receptor status of bladder carcinoma cells and the response to erlotinib [85] which demonstrated that even in absence of receptor mutations erlotinib showed potential as a therapeutic agent.

HER2/neu

HER2/neu is another transmembrane tyrosine kinase growth factor receptor which is overexpressed in urinary bladder carcinoma. In contrast to breast carcinoma, where HER-2/neu gene amplification and receptor overexpression could be correlated and had prognostic and therapeutic values, varying results have been reported for bladder cancer, and the prognostic significance is discussed controversially [86-92]. However, trastuzumab (Herceptin®), a recombinant monoclonal antibody against HER2/neu, which gained FDA approval for metastatic breast cancer, is currently under investigation in combination with conventional chemotherapy. In a multicenter phase II study with 44 patients with HER-2/neu -positive advanced urothelial carcinoma, Hussain et al. [93] investigated the safety and efficacy of trastzumab, carboplatin, gemcitabine, and paclitaxel. Treatment was considered feasible with a response rate of 70% and a median survival of 14 months which was favorable as compared to results with gemcitabine/carboplatin. Ongoing studies will concentrate on the true contribution of trastuzumab and, in general, on finding the best way to detect HER2/neu in urothelial cancer as immunohistochemistry (IHC) and fluorescent in-situ hybridization (FISH) do not correlate to the same extent.

Dual EGFR/HER2

Dual EGFR/HER2 inhibition is achieved with GW-572016/lapatinib (Tykerb®), which is also an orally active small molecule TKI. Being tested in a phase II study in pretreated bladder cancer patients, a median time of progression of 8.6 weeks was assessed, which was comparable to conventional second-line therapies [94].

3.2. Ras signalling pathway

Ras is a signal transduction protein which controls signaling pathways that are key regulators of several aspects of normal cell growth and malignant transformation [95]. As Ras mutations have been found in 30-40% of urothelial malignancies [96], blocking the activation of the Ras family oncogenes by farnesyl transferase inhibitors was also tested in bladder cancer treatment. Winquist et al. [97] evaluated the antitumor activity of SCH 66336/lonafarnib in a phase II study with patients with previously treated transitional cell carcinoma (TCC). No single-agent activity was observed, and thus, an ongoing study concentrates on combined therapy with gemcitabine [78].

R115777/tipifarnib (Zarnestra®) is another farnesyl transferase inhibitor. As compared to lonafarnib, a phase II trial in patients with metastatic TCC of the urothelial tract, did not show single-agent activity [98]. At this, studies are still continued. Another approach combines trastuzumab with tipifarnib for bladder cancer treatment [78].

3.3. Phosphatidylinositol-3-kinase signalling pathway

The mammalian target of rapamycin (mTOR) is a protein kinase of the phosphatidylinositol-3-kinase (PI3K)/Akt signalling pathway involved in cell growth, cell motility, cell survival, protein synthesis, and transcription [99]. As dysregulation of the mTOR pathway has been found in many human tumors, mTOR inhibitors are under investigation for cancer treatment. Currently, RAD001/everolimus (Certican®), which is

approved in Europe for drug-eluting coronary stents as an immunosuppressant to prevent restenosis, is under investigation in bladder cancer. In a phase II study the response rate and the effects of everolimus will be evaluated [78].

3.4. Cell cycle regulators

Cyclin-dependent kinases (CDKs) are main regulators of cell cycle progression, and thus inhibition results in cell cycle arrest and even in apoptosis. Alvocidib (flavopiridol, HMR 1275, L86-8275) is a semi-synthetic flavon which was the first cyclin-dependent kinase inhibitor in human clinical trials [100]. Evaluation in bladder cancer was performed by Chien et al. [101], who elucidated the effects of flavopiridol on normal urothelial cells, immortalized urothelial cell lines, and bladder cancer cell lines. They showed that flavopiridol is capable of inducing G2/M arrest, growth inhibition, and a modest level of apoptosis. Wirger et al. [102] additionally examined the toxicity and efficacy of flavopiridol in a rat bladder cancer model. Accordingly, this agent could be useful for bladder cancer therapy as 7 out of 12 rats were tumor-free and for the remaining tumors a tendency to lower stage and grade was demonstrated.

3.5. Proteasome

The ubiquitin-proteasome system is also involved in the regulation of cellular processes such as cell cycle and division, regulation of transcription factors, and cellular quality control by degradation of intracellular proteins. Thus, aberrations can play an important role in tumorigenesis and, consequently, the system has become a target for anticancer strategies [103].

PS-341/bortezomib (Velcade®), a specific and reversible inhibitor of the proteasome, is FDA approved for use in multiple myeloma. Kamat et al. [104] demonstrated for 253JB-V bladder cancer cells, that bortezomib inhibits cell growth and augments the growth

inhibitory effects of gemcitabine. Besides accumulation of p53, p21 and suppression of cyclin-dependent kinase 2 activity, antiangiogenic effects by inhibiting MMP-9, IL-8, and VEGF were also reported. Recent clinical studies further investigate the efficacy of bortezomib therapy for patients with advanced or metastatic transitional cell cancer of the bladder, the renal pelvis, or the ureter [78].

3.6. DNA hypermethylation

Aberrant DNA methylation has been shown to be a key survival mechanism in cancer. Inhibitors of DNA methylation, such as 5-azacytidine (5-Aza-CR), 5-aza-2-deoxycytidine (5-Aza-CdR) and zebularine, act through reactivation of the expression of genes that have undergone epigenetic silencing [105]. For bladder cancer therapy, Bender et al. [106] evaluated the effects of 5-Aza-CdR on human cancer cell lines (bladder transitional carcinoma- and colon carcinoma-derived cell lines) and human fibroblast cell strains. Results revealed suppression of cell growth in the tumor cell lines which was associated with reactivation of growth-regulatory genes silenced by de nova methylation. Recently, Cheng et al. [107] showed an antitumor effect of zebularine on human bladder carcinoma cells transplanted in BALB/c nu/nu mice after oral administration supporting the feasibility in cancer treatment.

3.7. Telomerase

Human telomerase reverse transcriptase (hTERT) is an essential component in the telomerase complex controlling telomerase activity and is highly expressed in bladder cancer cells. Zou et al. [108] investigated anti-proliferative effects of small hairpin interfering RNA (shRNA)-targeted hTERT gene on bladder cancer cells and xenograft mice models. Results revealed down-regulation of hTERT expression, and decreasing telomerase activity leading to suppression of tumor growth. Gao et al. [109] also

observed enhanced apoptosis of cancer cells upon administration of hTERT antisense oligodeoxynucleotides followed by TNF-α.

3.8. B-cell lymphoma 2 (bcl-2)

Bcl-2 is an important anti-apoptotic factor, and overexpression has been reported in bladder cancer [110]. Bilim et al. [111] evaluated bcl-2 antisense phosphorothioate oligodeoxynucleotide (PODN)-mediated downregulation of bcl-2 expression on human bladder cancer cells. Treatment reduced the bcl-2 protein level and combined treatment with Adriamycin resulted in increased cytotoxicity. Another synergistic effect resulting in significant lower cell survival rates of bladder cancer cells was observable for combined treatment of bcl-2 antisense oligonucleotide and cisplatin [112]. Currently, a phase I/II trial aims to study the effectiveness of bcl-2 antisense oligonucleotide (G3139, oblimersen, Genasense®) in treating patients with solid tumors including bladder tumors [78].

3.9. Clusterin

Clusterin (apolipoprotein J), a glycoprotein that is associated with a variety of functions such as regulation of apoptosis, has been reported to be overexpressed in several human cancers [113]. Miyake et al. [114] evaluated whether antisense (As) oligodeoxynucleotide (ODN) targeting the clusterin gene could enhance the cytotoxic effect of gemcitabine in human bladder cancer KoTCC-1 cells. The treatment resulted in significantly enhanced chemosensitivity of KoTCC-1 cells and administration in a murine intraperitoneal bladder tumour implantation model significantly decreased KoTCC-1 tumour volume. OGX-011/curtisen is a second generation antisense molecule which blocks production of clusterin and is currently under investigation in a phase I study in combination with docetaxel for therapy of patients suffering from metastatic or locally recurrent solid tumors (bladder, breast, kidney,…).

3.10. Survivin

Survivin is an apoptosis inhibitor that is up-regulated in many malignancies including bladder cancer. In this context, Fuessel et al. and Ning et al. [115,116] evaluated the efficacy of anti-survivin As-ODNs for bladder cancer treatment. According to tests with diverse bladder cancer cell lines, the survivin- directed As-ODNs down-regulated the expression of the protein and inhibited proliferation.

3.11. Tumor suppressor genes

Alterations in p53 and pRb, the products of the chromosomes 17p13 TP53 and 13q14 retinoblastoma (Rb) tumor suppressor genes, occur in almost 50% and 33% of bladder cancers, respectively, and are associated with advanced stages and higher grade of disease [117]. Thus, therapeutic strategies for restoration of the normal function are under investigation.

p53 gene-based therapy

An encouraging approach is the adenovirus-mediated transfer of wild-type human p53. The intratumoral injection of an adenoviral expression vector encoding wild-type p53 (rAd/p53 or SCH 58500) in patients with invasive bladder cancer [118] revealed biological activity of the treatment without any dose-limited toxicity. In a phase I study Pagliaro et al. [119] investigated the feasibility, safety, and biological activity of another adenoviral vector containing the wild-type *p53* gene, namely Ad5CMV-p53 (INGN201, Advexin®), in patients with locally advanced TCC of the bladder. Repeated installations were safely administered, but the efficiency of gene transfer turned out to be improvable as specific transgene expression was only observed in 2 out of 7 patients. Enhancement of the activity of Ad5CMV-p53 was observed in combination with cisplatin in a study with bladder cell lines [120] as well as in combination with As-ODN-targeting clusterin gene in a human bladder cancer model [121]. Another approach is the use of recombinant

vaccinia virus (rVV) as a vector for p53. In an orthotopic murine model, Fodor et al. [122] evaluated the efficacy of rVV-TK-53. The treatment resulted in decreased tumor incidence and in 33% survival, whereas the delivery using buffer or empty vector did not show any survival benefit.

pRb gene-based therapy

In preclinical studies with non-small cell lung carcinoma and bladder carcinoma cells, Xu et al. [123] evaluated the activity of RB94, a more potent tumour growth suppressor than the normal protein (Rb110), in a replication-deficient adenoviral vector (AdCMVpRB94). Gene therapy resulted in regression of the tumour of both Rb-negative and Rb-positive tumour cells and the pRB94, when overexpressed in tumour cells via replication-deficient adenovirus vectors, was evidently more potent in tumor suppression than the full-length RB protein. Generally, increasing emphasis for cancer gene therapy lies on the development and use of nonviral delivery methods which lack immunogenicity or biohazardous potential. In this context, Pirollo et al. [124] developed a systemically administrable, nanosized liposome delivery system with a tumor-targeting moiety, either the transferrin (Tf/Lip/RB94) or the anti-transferrin receptor single-chain antibody fragment (TfRScFv/Lip/RB94). Using an RB-negative human bladder carcinoma cell line, transfection with Tf/Lip/RB94 significantly sensitized cells for the chemotherapeutic agent. In mice bearing subcutaneous bladder tumors, the combination of systemically given Tf/Lip/RB94 or TfRScFv/Lip/RB94 plus gemcitabine resulted in significant tumor growth inhibition and/or regression as well as induction of apoptosis. A phase I study is aimed to evaluate this system in patients with metastatic RB-negative bladder cancer.

3.12. Histone deacetylase

Histone deacetylation is associated with transcriptional repression, including a decrease in the expression of tumor suppressor genes. Histone deacetylases (HDACs) are

overexpressed in various types of cancer, making them a potential target for cancer treatment. As HDACs also exert influence on the regulation of non-histone proteins such as heat shock protein 90 (Hsp90), p53, tubulin, Rb, and E2F1, HDAC-inhibitors seem to have a broad anti-cancer potential [125].

In terms of bladder cancer treatment, a fundamental study was performed by Canes et al. [126]. They investigated the effects of sodium butyrate (NaB) and trichostatin A (TSA), two histone deacetylase inhibitors, on bladder carcinoma cells and evaluated the inhibitory activity of TSA on cell growth. In a clinical trial, vorinostat (suberoylanilide hydroxamic acid (SAHA), NSC 701852, Zolinza), a small molecule inhibitor of HDAC and FDA approved for the treatment of cutaneous T cell lymphoma, was applied to 37 patients with advanced cancer [127]. Results revealed safe administration of the agent at doses that inhibit HDAC activity in-vivo, and 4 (2 lymphoma and 2 bladder) patients had objective tumor regression with clinical improvement in tumor related symptoms. Currently, a phase I study of vorinostat in combination with doxetaxel in patients with advanced and relapsed malignancies including bladder cancers is under investigation [78].

Romidepsin (FK228, depsipeptide, FR901228), another HDAC inhibitor, significantly inhibited growth of TCC tumor in xenograft models [128]. An ongoing phase II trial deals with the activity of romidepsin in patients with advanced cancer of the urothelium including that of the bladder.

3.13. Angiogenesis

Angiogenesis, the process of new blood vessel formation from preexisting ones, is one of the fundamental steps in the development of metastatic bladder cancer, and therefore serves as a sound target for cancer therapy. Among the angiogenic factors, the most prominent one involved in bladder cancer is the vascular endothelial growth factor (VEGF). Binding to the receptor results in stimulating cell growth and proliferation, two key features for the development of new blood vessels, and increased vascular

permeability, which may also contribute to angiogenesis and tumour growth [129]. The function of VEGF in vessel formation is complemented by the plateled-derived growth factor (PDGF) and thus, PDGF signaling can indirectly regulate angiogenesis [130]. Another important factor is the basic fibroblast growth factor (bFGF), which provides endothelial cells and smooth muscle cells with signals for proliferation and survival [131]. In addition to the important role of angiogenic factors for diagnosis and prognosis of bladder cancer [132, 133], they as well as their receptors are also subject of therapeutic strategies.

As blockade of the VEGF receptors has shown to decrease Ras signaling and DNA synthesis in bladder cancer cell lines [134], *DC101*, an *anti-VEGFR monoclonal antibody*, was applied to mice with metastatic human bladder carcinoma [135]. In this study, a significant anti-tumour efficacy was observed, especially when combined with paclitaxel. Another study of the same authors dealt with the effects of *DC101* on receptor phosphorylation and apoptosis and revealed that agents targeting a single receptor may not be sufficient to completely inhibit tumor angiogenesis [136]. *Bevacizumab (Avastin®)* is another recombinant humanised monoclonal antibody interacting with VEGFR and is FDA approved for the first-line treatment of metastatic colorectal cancer in combination with an intravenous 5-fluorouracil-based regimen. Currently, a study combining cisplatin, gemcitabine and bevacizumab for therapy of metastatic transitional bladder cancer is under evaluation [78].

The VEGF receptor activity can also be inhibited by catalytic RNA molecules known as ribozymes, which can downregulate VEGF receptor function by specifically cleaving the VEGFR1-mRNA. A study in patients with advanced solid tumors including [137] bladder cancer and administration of *RPI.4610* (*Angiozyme*), a stabilised ribozyme, in combination with carboplatin and paclitaxel revealed a complete response. The focus was set on the safety and the pharmacokinetics of *RPI.4610* in this combined therapy and the results indicated safe administration without substantial pharmacokinetic interactions.

Targeting VEGF instead of its receptor, *VEGF Trap* represents a potent angiogenesis inhibitor that consists of partial sequences of human VEGF receptor VEGFR1 and

VEGFR2 extracellular domains fused to the Fc portion of human immunoglobulin γ [130], which binds and inactivates VEGF with a high affinity. To date, a phase II trial is studying the side effects and the activity of *VEGF Trap* upon treatment of patients with recurrent, locally, advanced or metastatic bladder cancer [78].

BAY 43-9006/sorafenib (*Nexavar*®) is an orally adminstrable small molecule tyrosine kinase inhibitor, which is FDA approved for treatment of metastatic renal cell carcinoma. Inhibiting c-Raf, B-Raf, VEGFR2, VEGFR3 and PDGFR-B [138], it is currently under investigation for therapy of advanced and metastic bladder cancer [78]. *SU11248/sunitinib* (*Sutent*®) is another orally given, small molecule inhibitor of multiple tyrosine kinases, that targets PDGF, VEGFR, stem cell receptor factor (KIT), and fms-like tyrosine kinase 3 (FLT-3) [139]. It is FDA approved for the treatment of renal cell carcinoma and Imatinib-resistant gastrointestinal stromal tumour. In preclinical bladder carcinoma models, Sonpavde et al. [140] assessed the efficacy of sunitinib or cisplatin and in combination. Anti-tumour activity was observed as a single agent and the activity of cisplatin was enhanced. Recently, Bradley et al. [141] designed a randomised phase II trial evaluating the role of sunitinib as maintenance therapy in advanced urothelial cancer.

Endostatin protein is a naturally-occurring 20-kDa C-terminal fragment derived from collagen XVIII, which inhibits angiogenesis, and may interfere with the pro-angiogenic action of growth factors such as VEGF and bFGF [142]. Du et al. [143] investigated the efficacy of recombinant human endostatin to inhibit tumour growth in bladder cells. In contrast to tumour cells only proliferation of endothelial cells was inhibited, but in nude mice subcutaneous endostatin blocked angiogenesis and induced apoptosis in bladder cancer cells. Kikuchi et al. [144] evaluated lentivirus vector-mediated overexpression of endostatin and reported decreased vascularization and inhibition of bladder tumour growth. Thus, the application of endostatin seems to be a feasible concept, supported also by Schmidt et al. [145], who observed a distinct endostatin-binding pattern in bladder tumours as compared to benign tissue and malignant and benign kidney tissue.

TNP-470, an analogue of fumagillin and derived from *Aspergillus fumigatus*, is also a potent inhibitor of angiogenesis [146]. Various preclinical studies evaluated the efficacy for bladder cancer treatment of *TNP-470* alone [147-149] or in combination with chemotherapeutics [150,151]. In general, they demonstrated the potential of *TNP-470* to inhibit tumour growth and a synergistic antitumour effect with gemcitabine [151]. However, clinical data for bladder cancer are not available yet.

NF-κB is a protein complex, which acts as a transcription factor regulating the expression of pro-angiogenic molecules. In a study with human TCC cell lines, Karashima et al. [152] showed that *NF-κB* mediates neo-angiogenesis and proliferation via interleukin-8 which renders *NF-κB* a potential molecular target for bladder cancer therapy.

Targeting bFGF, Inoue et al. [153] investigated adenovirus-mediated *antisense bFGF* gene therapy in mice with human bladder carcinoma. They successfully inhibited tumour growth by downregulating the expression of bFGF and MMP-9 which resulted in decreased tumour cell proliferation and enhanced apoptosis of tumour and endothelial cells.

3.14. Glycocalyx

In common with all eukaryotic cells, the surface of bladder cells is covered by a dense layer of complex carbohydrates collectively known as the “glycocalyx”. Biorecognitive proteins such as lectins are capable of detecting and binding to these carbohydrate moieties at the cell surface in a highly specific manner. Due to this carbohydrate-specific interaction and alteration of the glycosylation pattern of cells upon malignant transformation [154], lectin-mediated targeting might be an encouraging approach towards site specific antitumour therapy. At this, binding to and even uptake in colon cancer cells was demonstrated for prodrug formulations as well as nanoparticulate drug carriers grafted with certain lectins to render these delivery systems targeted [155,156]. Plattner et al. [157] evaluated the cytoadhesive and cytoinvasive properties of several

plant lectins for a human bladder cancer cell line and prescreened wheat germ agglutinin as potential target vehicle for functionalized drug delivery systems.

4. Further Approaches

The matrix metalloproteinase (MMP) family of extracellular proteinases is regarded as a critical factor assisting tumour cells during metastasis by degrading the extracellular matrix [158]. A key role in this process is featured by MMP-2 and MMP-9, which mainly fragment the basement membrane type IV collagen. Recently, a study with *Magnolia officinalis* was performed [159] to investigate the anticancer activity to urinary bladder cells. A suppressed expression of MMP-2 and MMP-9 was monitored and the treatment of mice with BBN-induced urinary bladder tumors revealed growth inhibition. However, preliminary results from clinical trials aimed at therapy of various cancers have been disappointing [160], and thus the numerous functions of MMPs must be further investigated in order to find out effective targeting strategies.

Another target of future interest might be the Hsp90, which has been found to be overexpressed in several tumors including those of the bladder and is associated with progression of pathogenic cellular transformation [161,162]. Several Hsp90 inhibitors have entered clinical evaluation, which showed successful targeting, but advances are required to increase clinical outcomes in a wider range of cancers [163].

An interesting therapeutic approach is also the use of magnetic targeted carriers, which are microparticles formed from metallic iron and activated carbon. The iron provides magnetic manipulation and the carbon component allows absorption of doxorubicin (MTC-DOX). After intravesical instillation, targeting is achieved with the use of an externally placed magnet. Leakakos et al. [164] demonstrated for normal swine bladders

that MTC delivery allowed a greater exposure and site specific deposition of the drug in comparison to doxorubicin alone, but the proof of principle for the treatment of bladder cancer is still an open task.

Moreover, cell-penetrating peptides (CPP) or protein transduction domains (PDT) have become widely used vehicles for the cellular import of molecules, as they have been shown to be internalized in most cell types and can transport numerous types of drugs into cells including small-molecule pharmaceuticals, therapeutic proteins, and antisense oligonucleotides [165]. Schwarze et al. [166] synthesised a 15-oligomer peptide containing an NH_2-terminal 11-amino PDT from the human immunodeficiency virus TAT protein. The administration resulted in delivery of the biologically active fusion protein to all tissues in mice, including the brain. This lack of specificity might be problematic in case of systemic use, nevertheless, for intravesical therapy it could be of further interest.

Regarding bladder cancer treatment, also improved immunotherapeutic strategies have to be mentioned. The purpose of immunotherapy is to stimulate the immune system against tumour cells via either passive or active immunisation. A promising approach is direct induction of cytokines such as IL-2, IL-12, and interferons into cancer cells. The feasibility of an intravesical liposome-mediated IL-2 gene transfection was evaluated from Horiguchi et al. [167] in an orthotopic mouse bladder cancer model. Results revealed inhibited tumor growth and prolonged survival, which was enhanced by addition of subsequent B-7.1 (a surface immune molecule) gene-modified tumor vaccines [168]. Administration of IL-12 was investigated by O´Donnell et al. [169] in mice with transitional cell carcinoma of the bladder. Treatment resulted in prolonged survival and cured subjects (75%) at the highest IL-12 dose, and anti-tumor effects were observed after both systemic and intravesical administration. A preclinical study was conducted by Lee et al. [170] to examine the efficacy of murine IL-12 DNA vaccine and recombinant

BCG DNA vaccines in a murine bladder tumour model. The combination of both vaccines resulted in a high serum interferon-γ level, which promotes Th1 type of immune responses, and was associated with a significantly higher survival rate than that of mice treated with either vaccine alone or that of control mice. High expression of IL-12 can also be induced with AdCD40L gene therapy [171]. CD40L is a type II transmembrane protein belonging to the tumour necrosis factor superfamiliy, and the gene was transferred by adenoviral vectors to mice with orthotopic bladder cancers [171]. A systemic tumour-specific immune response was stimulated and 60% of mice with pre-established tumours were cured. Efficient gene delivery and expression of interferon α-2b protein has been achieved using a recombinant adenovirus gene delivery system (rAd-IFN) together with the small molecule excipient syn3 [172]. Results revealed increasing exposure of IFN and significant tumour reduction in a mouse model with human bladder cancer.

Finally, the role of radiochemotherapy (RCT) is under discussion in bladder cancer treatment. As radiotherapy (RT) alone has not shown to be superior to other conservative treatments [173], several studies have investigated the role of combined RT with chemotherapy to improve the outcome for patients [174-177] and suggested higher response rates and improved survival. Current approaches also concentrate on improved strategies in combination with hyperthermia [178] or inhibition of oncogene products such as HER2/neu in combination with RCT using paclitaxel and trastuzumab [179].

5. Expert opinion

Given the high tendency to recur together with the ever-present possibility to progress, successful treatment of bladder cancer is still a challenge for urologists and oncologists. Until now the primary goal has been to eradicate the local and any potential micrometastatic disease and at the same time to maintain the highest quality of life without compromising survival. Apart from surgical approaches, current treatment modalities comprise primarily non-specific immuno- or chemotherapy, but the response is often insufficient. Due to the physiology of the urothelium efficacy of treatment is governed by two crucial factors: the permeability of the bladder wall and the residence time of the API to maintain an adequate concentration gradient for passive diffusion. Improved permeability could be achieved via chemical and physical approaches also with good clinical results. However, all of these methodologies rely on non-specific permeation enhancement which might provoke some adverse side effects.

As to the residence time, conventional formulations typically stay in the bladder for only a short time of about 2 hours. Since a sufficiently high concentration gradient represents a crucial parameter for passive diffusion and thus efficacious treatment, a constantly elevated API-level in the bladder for a prolonged period would be desirable. Among the vehicles used for intravesical therapy colloidal carriers might be of particular importance especially in future. If these particles are refined with additional bioadhesive properties appropriate sustained release characteristics together with an often essential improve in stability of the API might be feasible.

However, all these modalities lack any clear discrimination between healthy and diseased tissue. Since the time of Paul Ehrlich pharmaceutical technologists all over the world dream of the “magic bullet”, a drug delivery system that has the ability to specifically target diseased cells. Especially in the field of chemotherapy with its numerous and severe side effects, site specific delivery and action of the API without compromising non-target cells are profound prerequisites for a more convenient regimen. Molecular

targeting might be a step forward in this area. Based on the increased understanding of the biological mechanisms underlying bladder carcinogenesis and tumour progression, various potential molecular targets have been identified and evaluated in preclinical studies, such as the signalling pathways playing a crucial role in the regulation of cell growth. Among others, the receptors of epithelial growth factor, which were found to be overexpressed in bladder cancer cells, have been addressed as attractive targets. Though preclinical data suggested positive aspects, the clinical benefits remain limited. This might also be due to the fact that EGFR expression is varying and there is often no clear correlation between overexpression of these receptors and tumour prognosis in bladder cancer. Possibly, response rates could be improved by an optimised patient selection using predictive molecular markers. According to the literature, application of specific inhibitors affecting the signalling pathway in a single-agent therapy seems to be generally less effective as compared to combination regimens using either chemotherapeutics or biologic agents that target a different part of the tumourigenetic cascade. At this, further therapeutic improvements might be achieved using sophisticated drug delivery systems instead of a simple parallel application. These systems might consist of nanoparticles that contain the cytotoxic agents in their core and are decorated with a corona of biologicals which guarantee the specific targeting.

Another approach towards targeted therapy relies on strategies acting at the genetic level. Various preclinical in-vitro and in-vivo studies suggested the principal feasibility of gene therapy for bladder cancer treatment. Currently, several phase I/II trials using either As-ODNs for suppression of oncogenic genes or tumour supressor genes for restoration of adequate cell cycle regulation are performed with promising results. Due to the rather easy access of the diseased tissue via intravesical instillation adequate delivery of the therapeutic genes to the cells of interest seems to be a manageable task making gene therapy a practicable option for treatment of bladder cancer. However, refinement of vector systems remains a challenge for future research in order to increase the transfection efficacy and its specificity. Despite encouraging results, a final decision about the potential of gene therapy in bladder cancer cannot be given yet.

Part I: bladder

With special emphasis on advanced bladder cancer also anti-angiogenic approaches might hold promise. Prominent targets in this area include either proangiogenic factors such as VEGF and bFGF or the corresponding receptors. Some of the strategies directed against angiogenesis of bladder tumours revealed their potential already in clinical trials and might become even more important in future.

An interesting alternative strategy towards improved selectivity of chemotherapy in bladder cancer might represent the application of lectins. Offering a clear discrimination between healthy and diseased cells, the advantage of the glycotargeting approach relies not only on the specific enrichment of lectin at the surface of malignant cells but also on the fact that some lectins are able to actively transport conjugated drugs across the cellular membrane. That way, the chemotherapeutic agent might be accumulated directly in the cytosol of the targeted cancer cells, if the API and the targeter are linked in a proper way either as a prodrug formulation or a carrier system.

All in all, pure combination chemotherapy in bladder cancer treatment has reached a plateau. However, novel biological therapies appear as a silver lining on the horizon of bladder cancer therapy that will gain increasing interest in future due to their targeting facilities. The challenge to be faced in this area by investigators all over the world is how to intelligently combine controlled and targeted delivery approaches in order to gain maximal treatment efficacy and thus therapeutic benefit for the patient with minimal physiological stress. Given the current efforts this might already be achievable within our lifetime.

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Part II: chondrocytes

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Lectin binding studies on C-28/I2 and T/C-28a2 chondrocytes provide a basis for new tissue engineering and drug delivery perspectives in cartilage research

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Abstract

The present study was performed to evaluate the applicability of plant lectins as mediators of bioadhesion in cartilage research using human chondrocyte cell lines C-28/I2 and T/C-28a2. The bioadhesive properties of fluorescein-labelled lectins with different carbohydrate specificities were investigated by flow cytometry. Specificity of the lectin–cell interactions was ascertained by competitive inhibition using complementary carbohydrates. As compared to that of other lectins, the interaction between wheat germ agglutinin (WGA) and chondrocytic cells was characterised by remarkable cytoadhesion, adequate binding strength and a high degree of specificity for *N*-acetyl-glucosamine as contained in hyaluronan chains. We therefore suggest WGA to be a promising candidate for mediating bioadhesion to low-adhesive scaffolds in cartilage tissue engineering. Moreover, the WGA-association rate of C-28/I2 and T/C-28a2 cells was dependent on temperature indicating cellular uptake of membrane-bound WGA. Intracellular enrichment was confirmed by confocal microscopy. Equilibration of intracellular pH gradients with monensin resulted in the reversal of quenching effects indicating accumulation of WGA within acid compartments of chondrocytic cells. Thus, WGA might be internalised into chondrocytes together with hyaluronan via the CD44 receptor-mediated endocytosis pathway and accumulated within lysosomes. This physiological process could represent a feasible pathway to target WGA-functionalised drug delivery devices into chondrocytes.

Keywords: Chondrocytes; Lectins; Bioadhesion; Drug targeting; Tissue engineering

1. Introduction

Human articular cartilage is characterised by a sparse population of chondrocytes embedded in an abundant extracellular matrix. The pericellular matrix or glycocalyx, directly adjacent to the chondrocyte cell membrane, consists of large proteoglycan aggregates such as aggrecan or perlecan [1]. In the organisation of the pericellular matrix, hyaluronan chains play a key role serving as backbone structures for the fixation of chondroitin sulphate-rich aggrecans [2]. Association of hyaluronan with the cell surface is maintained by CD44 receptors known to be present in chondrocytes [1–3].

The diverse carbohydrate structures present in the glycocalyx of cells can serve as targets for biorecognitive proteins such as lectins. By definition, lectins possess at least one non-catalytic domain, which binds reversibly to specific mono- or oligosaccharides [4]. Due to these characteristics, lectins are broadly used in haematology and histochemistry to characterise glycoconjugate distribution in tissues and to obtain specific information on the localisation of carbohydrate moieties of macromolecules. Regarding cartilage, lectins have been used in histological studies to examine cartilage development, matrix synthesis and structural changes encountered in osteoarthritis [5–10]. The usefulness of lectins in detecting and studying carbohydrates in solution and on cell surfaces as well as the ubiquitous occurrence of lectins in nature provide a major stimulus for their continuing evaluation as feasible excipients

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for medical and pharmaceutical applications. However, to our knowledge, lectins have not been previously evaluated as mediators of bioadhesion in cartilage research.

Based on the observation that cell–matrix or cell–scaffold interactions are able to exert a relevant influence on the phenotype and the function of processed cells, the concept of bioadhesion has gained growing interest in tissue engineering [11,12]. Once damaged, articular cartilage is difficult to regenerate because of the low mitotic activity of chondrocytes and the avascular nature of this tissue [13]. In this regard, applications of biomaterials, tissue engineering technologies and other cell-based therapies have great clinical potential for treatment of otherwise nonhealable lesions of cartilage tissue. Therefore, innovative bioengineering techniques are being developed by a number of groups in an attempt to generate new cartilage, using scaffold materials and cultured cells [14–16]. The candidate scaffold materials have to meet multiple demands such as biocompatibility, low immunogenicity, biodegradability and mechanical stability. Unfortunately, the optimal scaffold has not been constructed so far. Among others, the inability of some materials to facilitate cell adhesion on their low-adhesive surfaces rules out their application as scaffold materials. Therefore, attempts have been made to promote cell adhesion onto the surface of synthetic polymer scaffolds by creating hybrid scaffolds using chondroitin sulphate [15] or collagen [17,18]. Moreover, a variety of bioactive molecules such as RGD containing peptides or gelatin have been linked to surfaces to improve bioadhesion and biocompatibility [19–22].

Vice versa, the concept of bioadhesion has been applied in drug delivery systems to target a molecule of interest to the intended site of action. In this context, the potential of lectins as mediators of bioadhesion for pharmaceutical drug delivery systems has been extensively outlined [4,23,24].

Despite the potential application of lectins in tissue engineering and drug delivery, a systematic lectin binding study on cultured human chondrocytes has not been reported. Therefore, the present study is aimed at assessing the binding characteristics and binding specificity of selected lectins with affinity for distinct carbohydrate structures on human chondrocyte cell lines C-28/I2 and T/C-28a2. Additional focus was set on the mechanisms involved in uptake and intracellular fate of wheat germ agglutinin (WGA).

2. Materials and methods

2.1. Materials

The fluorescein-labelled lectins from *Triticum vulgare* (WGA; molar ratio fluorescein/protein (F/P)=3.2), *Solanum tuberosum* (STL; F/P=2.9), *Dolichos biflorus* (DBA; F/P=5.7), *Arachis hypogaea* (PNA; F/P=4.7), *Ulex europaeus* (UEA; Ulex europaeus isoagglutinin I, F/P=4.0) and *Lens culinaris* (LCA; F/P=6.3) were purchased from Vector laboratories (Burlingame, USA) and contained >98% active conjugate and no free fluorescein. *N*-acetyl-D-glucosamine (GlcNAc), chitotriose, *N*-acetyl-D-galactosamine, D-galactosamine, α-L-fucose, D-mannose, *N*-acetyl-neuraminic acid (NeuAc), monensin, and 0.25% Trypsin–EDTA solution were from Sigma (St. Louis, MO, USA); all other chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany) unless otherwise specified.

2.2. Cell culture

The chondrocyte cell lines C-28/I2 and T/C-28a2 were cultivated in DMEM (GIBCO, Carlsbad, California) with 10% fetal calf serum (Biochrom AG, Berlin), 50 μg/ml ascorbate, and 0.2% gentamycin in a 5% CO_2/95% air atmosphere at 37 °C. Cells were subcultured at a subconfluent state by trypsination.

2.3. Determination of the lectin binding capacity of chondrocytic cells

After reaching confluence, C-28/I2 or T/C-28a2 cells were harvested by trypsination, collected by centrifugation, and processed immediately. 50 μl cell-suspension (3×10^5 cells) were incubated with 50 μl of a dilution series of the respective fluorescein-labelled lectin (3.125–100 pmol in PBS) for 5 min at 4 °C. Unbound lectin was removed by washing the cells twice using 100 μl PBS. Afterwards, the cells were resuspended in 1 ml particle free PBS in order to provide a single cell suspension suitable for flow cytometric analysis. For estimation of autofluorescence, control samples consisting of unlabelled cells were included in all experiments.

2.4. Specificity of the lectin–chondrocyte interaction

To assess the carbohydrate-specific binding of the lectins to C-28/I2 and T/C-28a2 chondrocytes, 50 μl cell suspension (3×10^5 cells) in PBS, 100 μl of a dilution series of the respective complementary carbohydrate, and 50 μl of a solution containing 25 pmol fluorescein-labelled lectin were processed as described above. The amount of inhibitory carbohydrates used for each lectin is displayed in Table 1.

2.5. Internalisation of cell-bound WGA

In order to evaluate the cellular uptake of WGA by energy-dependent transport processes, the influence of the incubation temperature with respect to the incubation period was elucidated. 50 μl cell suspension (3×10^5 cells) were incubated with 50 μl PBS containing 12.5 pmol fluorescein-labelled WGA as indicated above (pulse-incubation). After washing the cells 3 times with PBS, the cell suspension was further incubated in PBS for 0, 10, 60, 120, 180 or 240 min at either 4 °C or 37 °C (chase-incubation). Afterwards, the cell suspension was prepared for flow cytometry and the cell-associated fluorescence intensity was determined. After the addition of 40 μl monensin in ethanol (2.42 mM) and incubation for 3 min at room temperature, the cell-associated fluorescence intensity was analysed again in the flow cytometer. Upon 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.

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Table 1
Lectin characteristics and ranges of complementary sugars used in the competitive binding assays

Lectin	MW	Binds to	Which is found in	Inhibitory sugar	pmol sugar used (C-28/I2; T/C-28a2)
WGA	36000	GlcNAc-(β1,4)-GlcNAc>β-GlcNAc>NeuAc	Heparan sulphate, hyaluronic acid	*N*-acetyl-D-glucosamine	0.5–16
				Chitotriose	0.00098–0.03125
STL	100000	GlcNAc-(β1,4)-GlcNAc	Hyaluronic acid	*N*-acetyl-D-glucosamine	16–128
				Chitotriose	0.0156–0.5; 0.0039–0.125
LCA	49000	α-Man>α-Glc>α-GlcNAc; multiple sugar residues with Fuc and Man	Cartilage oligomeric matrix protein	D-mannose	1–32
UEA	63000	α-L-Fuc	Keratan sulphate	L-fucose	0.0078–0.25
PNA	110000	Gal-(β1,3)-GalNAc>GalN>Gal	Keratan sulphate chondroitinsultate	D-galactosamine	0.01563–0.5
DBA	120000	GalNAc-(α1.3)-GalNAc>α-GalNAc	Chondroitinsulfate	*N*-acetyl-D-galactosamine	

WGA, wheat germ agglutinin from *Triticum vulgare*; STL, Solanum tuberosum lectin from *Solanum tuberosum*, DBA, Dolichus biflorus agglutinin from *Dolichos biflorus*; PNA, peanut agglutinin from *Arachis hypogaea*; UEA, Ulex europaeus agglutinin from *Ulex europaeus*; LCA, Lens culinaris agglutinin from *Lens culinaris*; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetyl-D-galactosamine; Glc, glucose; GlcNAc, *N*-acetyl-D-glucosamine; Man, mannose; NeuAc, *N*-acetyl-neuraminic acid.

2.6. Flow cytometry

Cell-associated fluorescence intensities were analysed by flow cytometry using the manufacturer's supplied software. To exclude any dead cells, debris, and aggregates, viable cells were gated with regard to the adequate forward versus side scatter properties. From each sample, 3000 cells within this gate were analysed. Fluorescence emission of the fluorescein-labelled single cell population was measured at 525 nm (10 nm bandwidth) after excitation at 488 nm. Amplification of the fluorescence signals was adjusted to put the autofluorescence signal of unlabelled cells into the first decade of the four-decade log range. The mean fluorescence intensity (MFI) of the relevant cell population representing the binding of the conjugated lectins was used to quantify the presence of specific carbohydrate structures in the glycocalyx of chondrocytic cells. All MFI values were corrected for autofluorescence of cells by subtracting the MFI of the control population from the MFI of the labelled population. Flow cytometric measurements were performed using an Epics XL-MCL analytical flow cytometer from Coulter (FL, USA).

2.7. Confocal microscopy

T/C-28a2 cells were stained by incubating 50 µl cell suspension (3×10^5 cells) with 25 pmol fluorescein-labelled WGA in 50 µl PBS for 10 min at 4 °C and 37 °C, respectively. After removing the unbound lectin as described above, cells were immediately mounted for microscopy without fixation. Confocal images of fluorescent-labelled cells were obtained using a Zeiss Axiovert confocal microscope. Transmission light and fluorescence pictures were acquired at 60× magnification and the background offset of the green fluorescence detector was adjusted to eliminate any autofluorescence of unstained cells.

2.8. Cell viability tests

Prior to each experiment, viability of cells was determined by trypan blue exclusion and counting of viable cells using a Bürker–Türk hemocytometer. Viability of gated single cells upon incubation with lectins was determined by propidium iodide staining. As described above, 50 µl cell suspension (3×10^5 cells) and 50 µl of the respective lectin solution in PBS (25 pmol) were incubated for 5 min at 4 °C. After washing twice with PBS, the cells were resuspended in 1 ml particle free PBS and further incubated with 2.7 µl propidium iodide solution (150 µM in PBS) for 2 min at 4 °C and analysed by flow cytometry (excitation at 488 nm; emission at 595 nm; 10 nm bandwidth). Negative control samples were prepared with 50 µl PBS instead of the lectin solutions, positive control samples by incubating the cells with 100 µl methanol for 2 min at −20 °C.

2.9. Statistics

Statistical analyses were performed using the Microsoft Excel® integrated analysis tool. Hypothesis tests among two data sets were made by comparison of two means from independent (unpaired) samples (*t*-test). A value of $p<0.05$ was considered significant. Descriptive statistical analyses were performed using mean values and standard deviations (SD). All experiments were performed in triplicate.

3. Results

3.1. Lectin binding capacity of chondrocytic cells

To estimate the surface binding capacity of C-28/I2 and T/C-28a2 chondrocytes for plant lectins with different carbohydrate specificities, dilution series of fluorescein-labelled lectins were incubated with a fixed number of cells. The results of the saturation experiments are presented in Figs. 1 and 2. For comparison, MFI values of each individual lectin were related to an apparent conjugation number of 1 mol fluorescein per mole lectin. The resulting saturation curves increased as a function of increasing lectin amounts added and levelled off between 25 and 100 pmol lectin indicating saturable binding processes. The autofluorescence levels of both cell lines were in the range of 0.103±0.001. Regarding the binding of WGA, increasing lectin concentrations resulted in increasing cell-

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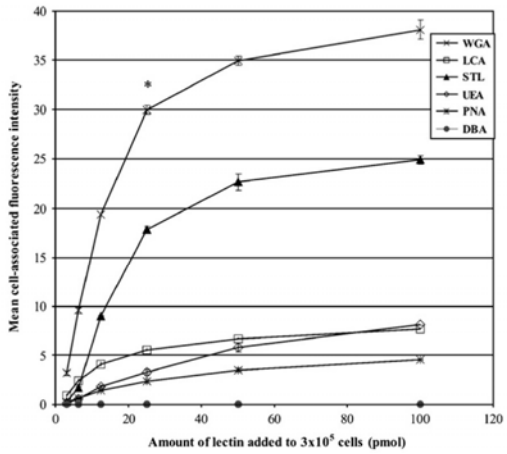

Fig. 1. Saturation analysis of lectin binding to C-28/I2 cells with fluorescein-labelled lectins related to an apparent F/P ratio of 1 (mean±SD, $n=3$). 3×10^5 cells were incubated with a dilution series of the respective fluorescein-labelled lectin (3.125–100 pmol in PBS) for 5 min at 4 °C. The significant difference ($p<0.05$) between WGA and other lectins at 25 pmol is marked with an asterisk.

bound fluorescence intensities between 3.17±0.23 and 38.1±0.98 in case of C-28/I2 and between 1.6±0.05 and 35±1.04 in case of T/C-28a2 cells. When 25 pmol lectin were applied in the binding studies on C-28/I2 cells, the obtained fluorescence intensity of WGA was 1.6, 5.4, 9.3, and 12.7 fold higher as compared to STL, LCA, UEA, and PNA, respectively (Fig. 1). These differences were statistically significant in each case ($p<0.05$). Fig. 2 clearly shows that these ratios were even more pronounced in T/C-28a2 cells. DBA only yielded very low MFI values below 0.01 in both cell lines which was scarcely above autofluorescence of cells.

3.2. Viability of chondrocytic cells

After trypsination, trypan blue exclusion yielded more than 90% (C-28/I2) and 85% (T/C-28a2) viable cells throughout the whole study. Viability of chondrocytes in the single cell population following incubation with lectins was assessed by propidium iodide staining. The used propidium iodide concentration resulted in a mean fluorescence intensity of 36.8±1.05 in case of methanol-treated cells. In contrast, upon incubation with 25 pmol lectin, the single cell population of both cell lines yielded MFI values <0.7 which are similar to values of PBS-treated cells (0.176±0.052) representing autofluorescence of cells. Since these values fall into the first decade of the four-decade log range of fluorescence intensity, it was concluded that the chondrocyte population used for the calculation of lectin binding capacity and specificity represented viable single cells. In the histograms, reduced cell viability after methanol treatment resulted in the appearance of a new population representing cellular fragments followed by the reduction of counts in the gate representing viable single cells. In general, the applied amounts of each lectin were tolerated well by the chondrocytic cells as represented by a high percentage of cells detected in the gate of single cells. However, upon incubation with increasing amounts of STL (3.125–100 pmol/3×10^5 cells) both cell lines showed a dose-dependent decline in viability characterised by an increasing number of cells counted in the methanol-related gate (data not shown).

3.3. Specificity of the lectin–chondrocyte interaction

Specificity of the interaction between plant lectins and the chondrocyte glycocalyx was investigated by competitive inhibition adding a dilution series of the corresponding carbohydrate (Table 1) to a constant amount of lectin (25 pmol). Depending on the amount of corresponding soluble carbohydrate added, the carbohydrate binding domain of the lectin is blocked partially and inhibited from binding to the cell surface. In the present study, the amount of cell-bound lectin generally decreased upon addition of increasing amounts of the corresponding carbohydrate indicating the specificity of the lectin–glycocalyx interaction. The percentage of decreasing fluorescence intensity as compared to a control sample without sugar representing 100% is shown in Table 2. For comparison of the data obtained, IC_{50} values were calculated from inhibition curves (not shown) of each lectin and displayed in Table 2. IC_{50} is defined as the amount of complementary carbohydrate which half-inhibits the binding of fluorescein-labelled lectins in the competitive binding assay.

The presented data demonstrate that with increasing amounts of GlcNAc added to C-28/I2 and T/C-28a2 cells the inhibition curves of WGA levelled off at 82.5% and 93.5%, respectively. This result indicates a high degree of specific binding of this lectin to the cell surface via its complementary carbohydrate structure. It is known that the specific binding of NeuAc to WGA is based on similarities in configuration of this sugar to GlcNAc at positions C-2 and C-3 of the pyranose ring [4]. However,

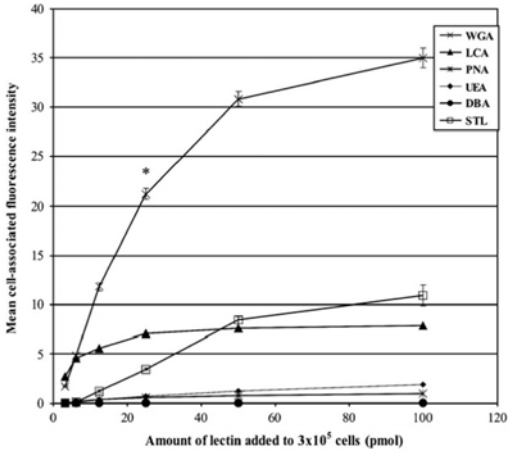

Fig. 2. Saturation analysis of lectin binding to T/C-28a2 cells with fluorescein-labelled lectins related to an apparent F/P ratio of 1 (mean±SD, $n=3$). 3×10^5 cells were incubated with a dilution series of the respective fluorescein-labelled lectin (3.125–100 pmol in PBS) for 5 min at 4 °C. The significant difference ($p<0.05$) between WGA and other lectins at 25 pmol is marked with an asterisk.

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Table 2
Competitive inhibition of lectin binding (25 pmol) to 3×10^5 C-28/I2 or T/C-28a2 cells by the addition of increasing amounts of complementary carbohydrate

	C-28/12			T/C-28a2		
Lectin+inhibiting sugar	Sugar [c] in μmol/reaction	% inhibition	IC_{50}	Sugar [c] in μmol/reaction	% inhibition	IC_{50}
WGA+GlcNAc	0.5	37.7±5.6	1.8	0.5	40.1±2.3	1.2
	1	40.5±3.1		1	45.4±1.1	
	2	52.7±2.1		2	64.9±0.6	
	4	65.5±0.8		4	78.7±0.4	
	8	75.0±0.8		8	86.3±0.8	
	16	82.5±0.4		16	93.5±0.3	
WGA+chitotriose	0.00098	23.8±2.3	0.007	0.00098	31.4±2.1	0.005
	0.00195	28.9±0.8		0.00195	35.0±1.6	
	0.00391	34.8±1.2		0.00391	44.9±1.5	
	0.00781	53.1±0.5		0.00781	59.8±3.8	
	0.01563	70.9±0.3		0.01563	73.1±1.0	
	0.03125	78.9±0.3		0.03125	81.6±1.4	
WGA+NeuAc	0.5	15.8±0.7	n.a.	0.25	8.1±0.6	n.a.
	1	35.2±4.2		0.5	16.8±3.5	
	4	35.1±6.7		2	41.7±1.5	
STL+GlcAcn	16	17.0±2.4	n.a.	16	38.2±1.5	58.5
	32	20.2±1.7		32	40.9±0.6	
	64	29.0±0.7		64	51.2±0.9	
	128	40.2±2.4		128	60.6±1.9	
STL+chitotriose	0.0156	18.2±0.8	0.08	0.0039	42.7±0.6	0.045
	0.03125	33.2±2.6		0.0078	52.1±1.8	
	0.0625	48.6±0.3		0.0156	62.9±1.4	
	0.125	65.2±1.0		0.03125	75.5±0.6	
	0.25	77.1±0.4		0.0625	82.9±0.5	
	0.5	87.8±0.4		0.125	89.4±0.8	
LCA+mannose	1	31.3±1.4	5	1	33.4±1.9	4.5
	2	36.5±0.8		2	38.3±1.3	
	4	46.8±0.8		4	48.6±1.3	
	8	58.8±0.3		8	61.7±0.7	
	16	69.7±1.6		16	73.1±1.2	
	32	79.8±0.8		32	83.9±0.3	
UEA+fucose	0.0078	52.6±0.9	0.175	0.0078	48.5±0.4	0.04
	0.01563	46.9±2.9		0.01563	48.4±2.5	
	0.03125	44.5±4.1		0.03125	48.3±0.5	
	0.0625	45.1±0.1		0.0625	55.2±1.7	
	0.125	46.7±1.6		0.125	63.4±0.5	
	0.25	55.6±1.5		0.25	78.2±0.5	
PNA+galactosamine	0.01563	40.2±0.5	0.185	0.01563	29.3±4.4	0.075
	0.0313	38.6±0.4		0.0313	32.9±0.9	
	0.0625	40.1±0.9		0.0625	46.9±1.9	
	0.125	44.6±0.9		0.125	58.9±1.1	
	0.25	54.7±0.9		0.25	69.6±1.8	
	0.5	72.1±0.1		0.5	85.6±1.7	

Results are presented as percent inhibition (mean±SD, $n=3$) as compared to control samples without sugar representing 100%. IC_{50} values represent the amount of complementary carbohydrate necessary for a 50% inhibition of the lectin binding (25 pmol) to 3×10^5 cells. n.a.: not assignable.

NeuAc binds to WGA with an affinity fourfold less than GlcNAc [4]. This ratio of binding affinities could be reproduced in our studies (Table 2). While 4 μmol NeuAc per reaction evoked a binding inhibition of 39.13±6.7% in C-28/I2 cells, a comparable inhibition (40.5±3.1%) could be achieved using 1 μmol GlcNAc. Using T/C-28a2 cells similar results were observed. Additional experiments using 8 and 32 μmol mannose yielded inhibition values of 32.3±8.2% and 31.9±2.4%, respectively, in case of C-28/I2 cells, and 16.01±1.03% and 16.13±7.01%, respectively, in case of T/C-28a2 cells. This lack of a concentration-dependent increase in binding inhibition together with the explicitly lower inhibition values of mannose compared to GlcNAc and chitotriose suggests that mannose inhibits WGA binding to chondrocytes solely via unspecific processes.

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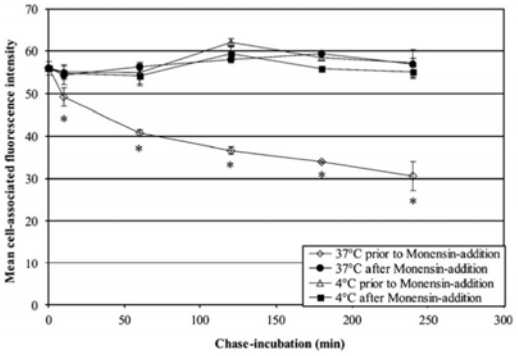

Fig. 3. Mean cell-associated fluorescence intensities of WGA-loaded C-28/I2 cells after incubation at both 4 °C and 37 °C for various time periods (mean± SD, $n=3$). Additionally, the mean cell-associated fluorescence intensities after addition of monensin (2.42 mM) are presented (mean±SD, $n=3$). Significant differences from the control sample (WGA, 5 min, 4 °C) are marked with asterisks ($p<0.05$).

The competitive binding assays using LCA and PNA revealed high inhibition values in the range from 72.1% to 85.6%. Interestingly, inhibition of STL binding to T/C-28a2 cells was achieved only by addition of excessive amounts of GlcNAc ($IC_{50}=58.5$). Due to the low solubility of GlcNAc, an inhibition of 50% could not be achieved using C-28/I2 cells. However, using chitotriose which is known to exhibit the 2×10^6-fold inhibitory activity of GlcNAc for STL an IC_{50} of 0.08 was obtained. The use of chitotriose as inhibitory sugar further allowed a comparison between the binding characteristics of WGA and STL on chondrocytic cells. Although both WGA and STL bind to similar structures (Table 1), the IC_{50} value of STL/chitotriose was one order of magnitude higher than that of WGA/chitotriose in both cell lines. Comparing the binding specificity of WGA and STL, about 80% of the WGA binding and about 88% of the STL binding could be inhibited by the complementary carbohydrate, presumably representing

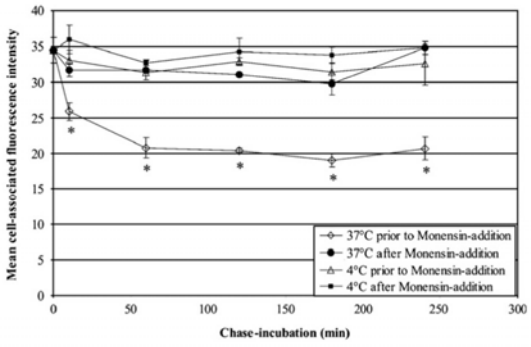

Fig. 4. Mean cell-associated fluorescence intensities of WGA-loaded T/C-28a2 cells after incubation at both 4 °C and 37 °C for various time periods (mean± SD, $n=3$). Additionally, the mean cell-associated fluorescence intensities after addition of monensin (2.42 mM) are presented (mean±SD, $n=3$). Significant differences from the control sample (WGA, 5 min, 4 °C) are marked with asterisks ($p<0.05$).

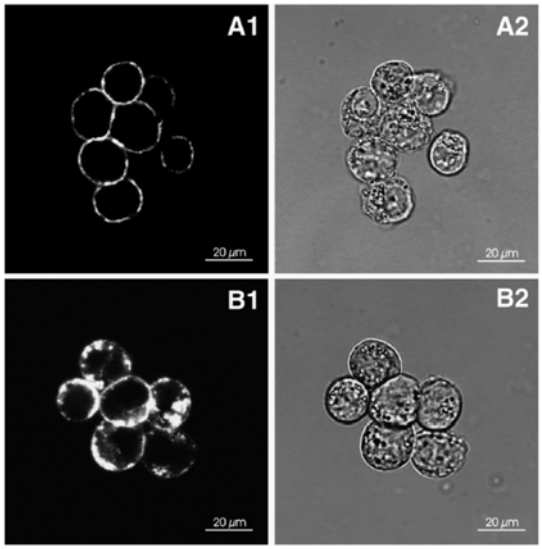

Fig. 5. Confocal images of T/C-28a2 cells after incubation with fluorescein-labelled WGA for 10 min at 4 or 37 °C (A1: 4 °C/fluorescence image; A2: 4 °C/transmission image; B1: 37 °C/fluorescence image; B2: 37 °C/transmission image). All images were acquired with the focus plane set to the middle of the cells. The cell diameter is about 20 µm.

the specifically bound fraction (Table 2). Together, these results suggest that the binding of STL to chondrocytic cells was characterised by slightly elevated specificity and much higher binding strength compared to that of WGA.

3.4. Internalisation of surface-bound WGA

The results of the internalisation experiments are displayed in Figs. 3 and 4 for C-28/I2 and T/C-28a2 cells, respectively. Upon chase-incubation of cells with surface-bound WGA for different intervals at 37 °C, a time-dependent decrease in fluorescence intensities was observed with both cell lines resulting in MFI values of 30.6±3.43 in case of C-28/I2 cells and 20.7±1.6 in case of T/C-28a2 cells after 240 min. As compared to a control sample measured immediately after the pulse-incubation with WGA for 5 min at 4 °C (55.9±1.6 in case of C-28/I2 and 34.5±1.7 in case of T/C-28a2), this decrease was significant at each time point ($p<0.05$). Upon treatment of WGA pretreated cells with monensin, the fluorescence values of both cell lines increased up to a level comparable to that found with control samples.

When the same assay was performed at 4 °C, no considerable differences between the MFI values prior and after monensin addition were observable over the whole period of time in both cell lines.

3.5. Confocal microscopy

The influence of the incubation temperature on the cellular distribution of WGA was visualised by confocal laser scanning

microscopy using T/C-28a2 cells (Fig. 5). All images were acquired with the focus plane set to the middle of the cells. After incubation with WGA at 4 °C for 10 min, the images exhibited solely a fluorescent ring around the cells. Intracellular enrichment of fluorescence was not detectable. In contrast, incubation with WGA at 37 °C for the same period of time resulted in a distinct dot-like accumulation of fluorescence intensity within the cells.

4. Discussion

The present study was performed to evaluate the applicability of plant lectins as mediators of bioadhesion in cartilage research using human chondrocytic cell lines as a reproducible model. In order to include the effects that different immortalisation techniques might exert, the experiments were performed on two cell lines of human origin, C-28/I2 and T/C-28a2 chondrocytes [25]. In comparison to C-28/I2 chondrocytes, T/C-28a2 cells yielded lower fluorescence intensities in the saturation experiments, indicating lower lectin binding. Only the surface binding rates of LCA were slightly higher in case of T/C-28a2 cells. All other lectins yielded significantly lower binding values on T/C-28a2 cells when 25 pmol lectin was applied ($p<0.05$). This finding is in agreement with the observation that the T/C-28a2 cell line proliferates more rapidly than the C-28/I2 clonal line. Since the expression of a differentiated phenotype in chondrocytic cell lines appears to be inversely related to their proliferative capacity [25], the reduced lectin binding rate in T/C-28a2 chondrocytes may reflect a smaller amount of pericellular matrix accumulated around these cells during culture.

When equal amounts of complementary carbohydrate were added in the competitive binding assays, the lectin binding rates were inhibited to a higher extent in T/C-28a2 cells indicating a higher fraction of specific binding of all lectins to this cell line. Moreover, the ratios between the IC_{50} values of C-28/I2 and T/C-28a2 cells were 1.5 in the case of WGA using GlcNAc, 1.1 in the case of LCA, 4.4 in the case of UEA and 2.5 in the case of PNA. Together, these findings suggest that T/C-28a2 cells presented smaller amounts of respective carbohydrate structures on their surface while, on the other hand, the interaction between the lectins and the T/C-28a2 cells was characterised by a higher degree of specificity but lesser intensity.

Even though the presented data indicate some differences between the binding rates of the cell lines in a direct comparison, the following key findings of our study were reproducible in both models.

4.1. Resulting perspectives for the use of lectins in tissue engineering of cartilage

Besides the fundamental interest in the basic principles of cell adhesion, the approach of engineering cell-material adhesion in artificial tissues remains a demanding challenge for biotechnologists. In tissue engineering, cell adhesion, spreading and growth onto surfaces are desired characteristics, for instance, of artificial cartilage grafts obtained by growing the patient's own chondrocytes on a scaffold providing sufficient mechanical stability and desired structural properties [18].

Despite some advantageous characteristics, candidate scaffolds such as chitosan, nylon or polyglycolic acid provide very limited cell attachment as compared to native fibres such as collagen [12,15,20,22]. Recently, Wang et al. demonstrated that cell attachment of fibroblasts on chitosan films was improved by the conjugation of WGA onto the chitosan surface [22]. In this way, cell densities on the scaffold were increased and biocompatibility was improved as determined by mRNA expression analysis of HSP 90 heat shock protein. Regarding the interaction of WGA and chondrocytes, a Japanese group has shown that chondrocytes exposed to soluble WGA became spherical [26]. This observation is remarkable since there is a well known relation between rounded cell shape and chondrocyte phenotypic expression. Moreover, a recent study showed that lectins were able to induce resistance to proteases and that WGA was among the 3 lectins yielding the highest effect [27].

The results of the present study indicate remarkable cytoadhesion as well as high binding affinity of WGA towards human chondrocyte cell lines. For both C-28/I2 and T/C-28a2 cells, the WGA binding capacity exceeded that of all other lectins by far (Figs. 1 and 2). As shown in Table 2, WGA also exhibited adequate binding strength and a high degree of specificity to its complementary carbohydrate GlcNAc which is one of the main structural elements of the chondrocyte glycocalyx. Furthermore, the examined amounts of WGA were well tolerated by the cells. In contrast, STL which binds with higher affinity to chondrocytes and which is specific for sugars similar to those bound by WGA caused a dose-dependent decrease in viability of both cell lines which might be due to its high binding strength observed. Regarding LCA, which actually showed a high degree of binding specificity, the low binding rates as compared to WGA suggest that D-mannose structures might not occur in sufficient amounts in the chondrocyte glycocalyx to act as bioadhesion targets. Similarly, the low binding rates and low IC_{50} values of other lectins such as DBA, UEA or PNA indicate the presence of only small amounts of complementary carbohydrate structures on the surface of the chondrocyte cell lines and are not likely to represent feasible bioadhesive agents.

In synopsis, WGA might be a promising candidate to mediate bioadhesion to low-adhesive scaffolds in cartilage tissue engineering approaches. However, the immobilisation of WGA onto biomaterial surfaces as well as the resulting effects of WGA-mediated cytoadhesion on chondrocyte function are beyond the scope of this paper and will be addressed in a forthcoming report.

4.2. Resulting perspectives for the use of lectins in drug delivery to chondrocytes

The application of lectins and lectin-like molecules to act as drug delivery adjuvants is currently an important trend [4,24,28]. In recent studies the enhancement of cytoadhesion, cytoinvasion as well as transcellular transport of WGA-grafted nanospheres was demonstrated using Caco-2 single cells and monolayers [29,30]. To explore the potential utility of lectins as

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bioadhesive tools for drug delivery to chondrocyte cells, the present paper outlines the interaction between lectins and C-28/I2 as well as T/C-28a2 chondrocytes. As both affinity and binding rate of WGA pointed to high cytoadhesiveness of this lectin (Figs. 1 and 2, Table 2), the WGA interaction with chondrocytic cell lines was elucidated in more detail. In this regard, the cellular fate of fluorescein-labelled WGA was investigated over a period of 4 h at 4 °C and 37 °C, respectively. As shown in Figs. 3 and 4 the incubation temperature exerted a significant influence on the measured MFI levels. The results at 4 °C indicate binding of the labelled lectin to the cell surface without providing evidence of the internalisation of cell-bound lectin at 4 °C. At 37 °C, however, fluidity of the cell membrane increases and energy consuming transport processes can occur. Consequently, lectin binding to the cell membrane may be followed by active transport of the lectin into the cell. Since the fluorescence emission of fluorescein is known to be reduced in an acidic environment, the decreasing quantum yield at 37 °C points to intracellular enrichment within acidic compartments such as lysosomes (Figs. 3 and 4). To verify this hypothesis, monensin was added to the cell suspension resulting in an inhibition of the quenching effects. Monensin represents a carboxylic ionophore which is known to catalyse the exchange of protons for potassium ions and therefore compensates the pH gradient between the cytoplasm and acidic compartments [31].

These results are in agreement with the findings of Hua et al. who demonstrated the internalisation of hyaluronan via receptor-mediated endocytosis in rat chondrosarcoma cells and bovine chondrocytes [2]. Using CD44 antibodies, the CD44 receptor was shown to play a major role in this process. It was further demonstrated that endocytosed HA accumulated in intracellular vesicles and was destined for degradation within the cell by lysosomal enzymes. In the present study, cytoinvasion experiments of cell-bound WGA using different temperature levels and monensin were additionally confirmed by confocal microscopy (Fig. 5). Interestingly, after incubation at 37 °C the cytoplasm was not stained uniformly but in a dot-like manner indicating a vesicular accumulation of the lectin.

In summary, the presented findings suggest that WGA which is specifically bound to GlcNAc structures of HA present in the glycocalyx of chondrocytes might get internalised into chondrocytes together with HA via the CD44 receptor-mediated endocytosis pathway. Inside the cells, the lectin could accumulate in lysosomes or the trans-Golgi complex. Since endocytosis of HA represents a mechanism for the maintenance and homeostasis of cartilage tissue [2], WGA-functionalised drug delivery devices could take advantage of this physiological pathway to target a drug of interest into chondrocyte cells.

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Lectin binding patterns reflect the phenotypic status of in vitro chondrocyte models

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Abstract In vitro studies using chondrocyte cell cultures have increased our understanding of cartilage physiology and the altered chondrocytic cell phenotype in joint diseases. Beside the use of primary cells isolated from cartilage specimens of donors, immortalized chondrocyte cell lines such as C-28/I2 and T/C-28a2 have facilitated reproducible and standardized experiments. Although carbohydrate structures appear of significance for cartilage function, the contribution of the chondrocyte glycocalyx to matrix assembly and alterations of the chondrocyte phenotype is poorly understood. Therefore, the present study aimed to evaluate the glycoprofile of primary human chondrocytes as well as of C-28/I2 and T/C-28a2 cells in culture. First, the chondrocytic phenotype of primary and immortalized cells was assessed using real-time reverse transcriptase polymerase chain reaction, immunofluorescence, and glycosaminoglycans staining. Then, a panel of lectins was selected to probe for a range of oligosaccharide sequences determining specific products of the *O*-glycosylation and *N*-glycosylation pathways. We found that differences in the molecular phenotype between primary chondrocytes and the immortalized chondrocyte cell models C-28/I2 and T/C-28a2 are reflected in the glycoprofile of the cells. In this regard, the glycocalyx of immortalized chondrocytes was characterized by reduced levels of high-mannose type and sialic acid-capped *N*-glycans as well as increased fucosylated *O*-glycosylation products. In summary, the present report emphasizes the glycophenotype as an integral part of the chondrocyte phenotype and points at a significant role of the glycophenotype in chondrocyte differentiation.

Keywords Chondrocytes · Glycophenotype · Phenotype · Lectins · *O*- and *N*-glycans

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Part II: chondrocytes

Introduction

Beside water, collagen fibers, and other components such as adhesion proteins and lipids, the extracellular matrix (ECM) of cartilage consists of numerous glycoproteins and proteoglycans which function in the maintenance of healthy cartilage (Knudson and Knudson 2001). The attachment of oligosaccharide chains to asparagine (*N*-linked) or serine/threonine (*O*-linked) residues represents a common post-translational modification of proteins potentially influencing biological processes such as apoptosis, inflammation, cell signaling, protection against proteolytic degradation, and protein folding (Knudson and Knudson 2001; Yang et al. 2007). Furthermore, the protein backbone of major cartilage proteoglycans is often associated with carbohydrate chains such as chondroitin sulfate, heparan sulfate, and keratan sulfate. Another key molecule required for matrix assembly is hyaluronan, a large linear glycosaminoglycan (GAG) composed of a repeating disaccharide (beta-D-glucuronyl-beta-D-*N*-acetylglucosamine) without core protein (Lee and Spicer 2000). It can either be secreted by chondrocytes into the ECM or stay associated with the plasma membrane to form a pericellular coat maintaining cell-substrate adhesion and reducing friction in cartilage. In general, carbohydrate structures are of fundamental importance for the functionality of cartilage since the high density of fixed charges from sulfated glycosaminoglycans (sGAG) is essential to maintain hydrostatic pressure and gives cartilage the resistance to bear loads.

In degenerative joint disorders such as osteoarthritis (OA), the breakdown of the cartilage matrix represents a major characteristic of the disease process. Typically, loosening of the tight collagen network as well as the loss of the interwoven proteoglycan aggregates determine the initiation and progression of cartilage degeneration (Aigner and Stove 2003). Being the only vital reactive elements within the tissue, chondrocytes play a pivotal role in the loss of cartilage function. It has been shown that increased matrix catabolisms as well as alterations of the matrix gene expression profile are important features of chondrocytes under osteoarthritic conditions (Martin et al. 2001). In vitro studies using chondrocyte cell cultures have increased our understanding of cartilage physiology and the altered chondrocytic cell phenotype in disease (Von der Mark et al. 1977; Martin et al. 2001; Stokes et al. 2002). In particular, immortalized chondrocyte cell lines such as the C-28/I2 and T/C-28a2 cell lines have facilitated reproducible and standardized experiments exploring chondrocyte-specific responses to stimuli such as cytokines or chondroprotective agents (Goldring et al. 1994; Oliver et al. 2005; Toegel et al. 2007b, 2008). Despite their high benefit, however, cell lines—as expected for cells in constant log-phase growth—were shown to differ from primary chondrocytes regarding mRNA profiles and other features of the chondrocyte phenotype (Finger et al. 2003).

Although carbohydrate structures appear of significance for cartilage function, the contribution of the chondrocyte glycocalyx to matrix assembly or alterations of the chondrocyte phenotype is poorly understood. Hence, the present study evaluates the glycoprofile of primary human chondrocytes in culture as well as the relation between chondrocytic phenotype and glycophenotype by comparing primary chondrocytes with the immortalized chondrocyte cell models C-28/I2 and T/C-28a2.

Materials and Methods

The fluorescein-labeled lectins from *Triticum vulgare* (wheat germ agglutinin (WGA); molar ratio fluorescein/protein (F/P)=3.1), *Solanum tuberosum* (STL; F/P=2.9), *Dolichos biflorus* (DBA; F/P=2.2), *Arachis hypogaea* (peanut agglutinin (PNA); F/P=5.2), *Ulex europaeus* (*Ulex europaeus* isoagglutinin I (UEA); F/P=2.7) and *Lens culinaris* (LCA; F/P=3.8) were purchased from Vector laboratories (Burlingame, USA) and contained >98% active conjugate and no free fluorescein. The Mx3000P QPCR instrument, the StrataScript First-Strand Synthesis System, and the Brilliant SYBR Green QPCR Master Mix were from Stratagene (La Jolla, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Isolation and culture of primary human chondrocytes. Human OA cartilage was obtained during total knee replacement surgery performed in patients with osteoarthritis (six patients; age 60–68; blood group 0 RhD positive) with informed consent and in accordance with the terms of the ethics committee of the Medical University of Vienna (EK-Nr.: 081/2005). Only cartilage specimens from femoral condyles and the tibial plateaus showing no visible signs of pathology were used. Primary chondrocytes were isolated following standard protocols using 0.2% collagenase II (Gibco, Carlsbad, CA). Primary chondrocytes were seeded at high density (10^5 cells/cm^2) and grown to confluence in an atmosphere of humidified 5% CO_2/95% air at 37°C using Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (Biochrom AG, Berlin, Germany), 2 µl/ml gentamycin, and 50 µg/ml ascorbate as culture medium (full medium). For all assays, only freshly isolated and seeded cells without subculturing were used.

Culture of immortalized human chondrocytes. The T/C-28a2 chondrocyte cell line was derived from primary cultures isolated from costal cartilage from a 15-yr-old

female by retroviral-mediated transfection with the large T antigen of Simian virus 49 (Goldring et al. 1994). C-28/I2 represents a cell line obtained after clonal expansion of the T/C-28 cells. Both cell lines retain chondrocytic morphology and maintain continuous proliferation in monolayer culture (Finger et al. 2003). C-28/I2 and T/C-28a2 cells were verified to be mycoplasma-free using the Venor GeM Mycoplasma PCR Detection Kit (Minerva Biolabs, Berlin, Germany), seeded at 9.3×10^3 cells/cm^2 and maintained in full medium as described for primary cells.

Lectin binding assay. Immortalized chondrocytes were cultured in 96-well microplates (Iwaki, Tokyo, Japan). Confluent monolayers were washed three times with 50 µl phosphate buffered saline (PBS) using a Columbus washer (SLT, Salzburg, Austria) prior to incubation with 50 µl of a dilution series of the respective fluorescein-labeled lectin (3.125–100 pmol/well in PBS) for 60 min at 4°C. Selected lectins, associated binding specificities, and target structures are presented in Table 1. Monolayers were washed again and the mean cell-bound fluorescence intensity (MFI) was determined using a Spectra Fluor fluorescence microplate reader (SLT) equipped with Biolise software. Fluorescence emission of the lectin-loaded cell monolayers with 50 µl PBS as supernatant was measured at 535 nm after excitation at 485 nm. Each experiment was performed in quadruplicate and control samples consisting of unlabeled cells were included.

Lectin binding patterns of cultured primary human chondrocytes were assessed using 50 µl of fluorescein-labeled lectins (25 pmol/well) following the same procedure.

Flow cytometry. The glycocalyx of primary human chondrocytes was assessed directly after isolation from cartilage specimens and after trypsin treatment (described below) following recently published protocols (Toegel et al. 2007a). Briefly, 3×10^5 cells were incubated with 25 pmol fluorescein-labelled lectins (50 µl) for 5 min at 4°C. After washing, the cells were resuspended in particle-free PBS and analyzed using an Epics XL-MCL analytical flow cytometer (Coulter, FL). The MFI of the relevant cell population representing the binding of the conjugated lectins was used to quantify the presence of specific carbohydrate structures in the glycocalyx of chondrocytic cells. All MFI values were corrected for autofluorescence of cells.

Specificity of the lectin-chondrocyte interaction. Confluent monolayers of C-28/I2 and T/C-28a2 cells cultured in 96-well microplates were washed three times with 50 µl PBS. Then, 100 µl of a dilution series of the respective complementary carbohydrate (Table 2) and 50 µl of a solution containing 25 pmol fluorescein-labeled lectin were added followed by incubation for 60 min at 4°C. After washing, the cell-bound fluorescence intensity was determined by fluorimetry. Each experiment was performed in quadruplicate and reference values accounting for the fluorescence maximum (0% inhibition) were determined using 100 µl PBS instead of the carbohydrate solution. Specificity of lectin binding to primary OA human chondrocytes was controlled using the respective complementary sugars and 25 pmol fluorescein-labeled lectin as described above. Hyaluronan oligosaccharides used to characterize the binding specificity of WGA were generated by a 24-h digestion of 20 mg hyaluronan (MW ~ 1,500 kD) with 439 U of bovine testicular hyaluronidase in 1 ml digestion buffer (0.1 M phosphate puffer, pH 5.3, 0.15 M NaCl) at 37°C. It has been reported that the final reaction product of this digestion consists mainly of tetrasaccharides (Takagaki et al. 1994).

The binding sites of WGA and PNA were further evaluated after enzyme-catalyzed hydrolytic removal of sialic acid residues. Confluent T/C-28a2 monolayers were treated with 100 µl neuraminidase (Type V; Clostridium perfringens; 0.05 and 0.1 U/ml in 0.2 M sodium acetate, pH 5.5) for 60 min at 37°C. Subsequently, the monolayers were extensively washed with PBS prior to labeling with 50 µl fluorescein-labeled WGA or PNA (50 pmol; 60 min; 4°C). After washing, cell-bound fluorescence intensities were determined by fluorimetry. Each experiment was performed in triplicate and control samples consisting of

Table 1. Sources, specificities, and possible target structures at chondrocyte cultures of lectins used

Lectin	Source	Binding specificity	Target structures
WGA	*Triticum vulgaris*/wheat germ	*N*,*N*′,*N*″-triacetylchitotriose > GlcNAc > sialic acid	Hybrid-type *N*-glycans, keratin sulfate, hyaluronan, complex oligoantennary glycans
STL	*Solanum tuberosum*/potato	Poly-*n*-acetyllactosamine	Keratin sulfate
LCA	*Lens cultinaris*/lentil	D-Mannose	High-mannose type *N*-glycan
SNA	*Sambucus nigra*/elder	Sialic acido2-6Gal−	Sialic acid caps of complex-type *N*-glycan
DBA	*Dolichus biflorus*/horse gram	GalNAc−	First step of *O*-glycosylation pathway
PNA	*Arachis hypogaea*/peanut	Galβ1-3GalNAc−	Unmodified *O*-glycan core 1
UEA	*Ulex europaeus*/gorse	Fucα1-2Galβ1-4GlcNAc−	Fucosylated *O*-glycan

Part II: chondrocytes

Table 2. Competitive inhibition of lectin binding (25 pmol/well) to confluent C-28/I2 or T/C-28a2 cell monolayers was performed by addition of increasing amounts of complementary carbohydrate

Lectin + inhibiting sugar	IC50	
	C-28/I2	T/C-28a2
WGA + GlcNAc	1.75	1.1
WGA + triacetylchitotriose	0.005	0.005
WGA + hyaluronan oligosaccharides	0.15	0.32
STL + GlcNAc	15	30
STL + triacetylchitotriose	0.02	0.01
LCA + mannose	2.5	15.3
SNA + sialyllactose	0.46	0.65
UEA + fucose	0.1	0.08
UEA + hyaluronan oligosaccharides	–	–
PNA + galactosamine	0.18	0.33

IC_{50} values represent the amount of complementary carbohydrate necessary for a 50% inhibition of the lectin binding to confluent cell monolayers. The concentration of hyaluronan oligosaccharides was calculated based on the molecular weight of hyaluronan of 1,500 kD prior to enzymatic digestion. Sialyllactose is commercially available as mixture of α-2,6- and α-2,3-isomers.

unlabelled cells were included. Additional controls were performed by substituting the neuraminidase with PBS.

Moreover, primary chondrocytes were treated with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) for 20 min at 37°C followed by washing with PBS. Then, flow cytometry using 25 pmol fluorescein-labeled WGA, *L. culinaris* agglutinin, and *Sambucus nigra* agglutinin was performed as described above.

Immunofluorescence and alcian-blue staining. Confluent monolayers of C-28/I2 cells, T/C-28a2 cells, and primary human OA chondrocytes on glass cover slides were washed with PBS and fixed with 400 µl methanol (10 min, −20°C) prior to incubation with mouse monoclonal primary antibodies against aggrecan, collagen type I, and collagen type II (Calbiochem, San Diego, CA) for 60 min at 37°C. Then, cells were washed using 1% bovine serum albumin (BSA) in PBS followed by incubation with a fluorescein isothiocyanate-conjugated antimouse-Ig secondary antibody (Dako, Hamburg, Germany) for 30 min at 37°C. Monolayers were rinsed again, embedded in FluorSave antifade medium (Calbiochem), and examined using a Nikon Eclipse 50i fluorescence microscope. Negative controls were prepared using 1% BSA/PBS instead of the primary antibodies.

Alcian blue 8GX was dissolved in 3% acetic acid or 0.1 M HCl to prepare 1% alcian blue solutions of pHs 2.5 and 1, respectively. C-28/I2 cells and primary human OA chondrocytes were seeded in triplicate into six-well plates and cultured as described above. Upon confluence, cells were fixed with 10% neutral buffered formalin for 10 min at room temperature. After washing twice with PBS, cells were incubated with 3% acetic acid for 10 min. The entire amount of GAG was stained with alcian blue solution at pH 2.5, whereas sGAG were stained with alcian blue solution at pH 1 (Kiernan 2006). After 30 min, cell monolayers were rinsed five times with PBS prior to extraction of alcian blue with dimethyl sulfoxide. Absorbance was measured in triplicate at 650 nm using a Tecan Infinite M200 microplate reader (SLT). Finally, absorbance values were normalized to equal DNA contents as evaluated using the CyQuant assay (Piana et al. 2007).

Quantitative. RT-PCR. C-28/I2 cells and primary human OA chondrocytes from three donors were seeded in duplicate into 12-well microplates. Upon confluence, total RNA was extracted using the NucleoSpin RNA II Kit (Macherey-Nagel, Dueren, Germany). Each sample was run on the Agilent 2100 Bioanalyzer Nano LabChip for quality control and quantification of total RNA prior to reverse transcription into cDNA using the StrataScript first-strand synthesis system. All quantitative reverse transcriptase polymerase chain reaction (qPCR) reactions for transcripts from collagen type I (COL1), collagen type II (COL2), and aggrecan (AGC) were performed in 25 µl reaction mixtures pursuing protocols published recently (Toegel et al. 2008). Expression levels were calculated by the equation $2^{-\text{deltaCt}}$ with respect to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as reference gene.

Statistics. Hypothesis tests among two data sets were made using paired or unpaired Student's *t* test. Statistics cross-comparing several study groups were performed using one-way analysis of variance with post hoc Tukey test. Values of $p<0.05$ were considered significant.

Results

Lectin binding patterns of isolated human chondrocytes. Figure 1 shows the binding level of isolated human chondrocytes for WGA, *S. tuberosum* lectin, *L. culinaris* agglutinin, *S. nigra* agglutinin, and PNA as assessed by flow cytometry ($n=6$ donors). Mean ratios between WGA and *L. culinaris* agglutinin, *S. nigra* agglutinin, *S. tuberosum* lectin, and PNA were 0.7, 1.2, 4.1, and 31.1, respectively. At this, *S. nigra* agglutinin, *S. tuberosum* lectin, and PNA yielded significantly lower binding values than WGA ($p<0.05$, paired *t* test). When primary chondrocytes were treated with trypsin-EDTA, a significant drop in binding of *L. culinaris* agglutinin and *S. nigra* agglutinin

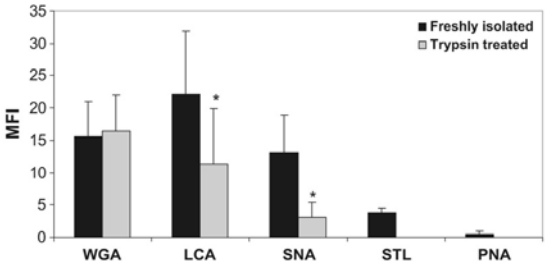


Figure 1. Flow cytometric analyses of lectin binding by primary chondrocytes (mean ± standard deviation, n=6) using fluorescein-labeled WGA, LCA, SNA, STL, and PNA. Chondrocytes isolated by collagenase II digestion of cartilage specimens and chondrocytes treated subsequently with 0.25% trypsin-EDTA (20 min, 37°C) are compared. Significant differences were determined using paired Student's t test and marked with *asterisks* (p<0.05).

was observed (Fig. 1; p<0.05, paired t test). In contrast, WGA binding sites were not affected by trypsinization.

Lectin binding patterns of human chondrocyte monolayers. The glycoprofile of primary chondrocyte monolayers was evaluated using seven fluorescence-labeled plant lectins (Table 1; Fig. 2). The ranking of bound lectins remained stable among the donors (PC1-3), with either WGA or *L. culinaris* agglutinin exhibiting the highest binding followed by *S. nigra* agglutinin, *S. tuberosum* lectin, PNA, *D. biflorus* agglutinin, and *U. europaeus* isoagglutinin I. The mean fluorescence intensities among the three individual donors were 589.3±88.9 for WGA, 393.7±109.9 for *S. tuberosum* lectin, 608.3±254.5 for *L. culinaris* agglutinin, 429.0±167.3 for *S. nigra* agglutinin, 10.7±4.6 for *U. europaeus* isoagglutinin I, 23.7±1.1 for *D. biflorus* agglutinin, and 119.7±54.2 for PNA. Mean ratios between WGA and *L. culinaris* agglutinin, *S. nigra* agglutinin, *S. tuberosum* lectin, PNA, *U. europaeus* isoagglutinin I, and *D. biflorus* agglutinin were 0.9, 1.5, 1.6, 5.7, 64.5, and 24.9, respectively. This indicates a comparable distribution pattern of lectin binding sites between freshly isolated cells and confluent chondrocyte monolayers.

The glycoprofile of immortalized C-28/I2 and T/C-28a2 cells was evaluated in more detail using rising concentrations of fluorescence-labeled plant lectins. Resulting binding curves increased as a function of increasing lectin concentrations and leveled off between 50 and 100 pmol/well lectin indicating saturable binding processes for both cell lines (Figs. 3 and 4). The figures illustrate that the fluorescence intensity of bound WGA was significantly higher than that of any other lectin (12.5–100 pmol WGA/well). At 50 pmol/well, WGA binding was 1.9, 2.5, 2.9, 3.5, 3.9, and 14.3-fold higher than that of *S. tuberosum* lectin, *L. culinaris* agglutinin, PNA, *U. europaeus* isoagglutinin I, *S. nigra* agglutinin, and *D. biflorus* agglutinin in the case of C-28/I2 cells (Fig. 3). In the case of T/C-28a2 cells, ratios of 2.0, 2.8, 7.9, 7.3, 2.8, and 43.6 were obtained. Together with a direct comparison of the respective fluorescence intensities, this indicates that binding levels of PNA, *U. europaeus* isoagglutinin I, and *D. biflorus* agglutinin were significantly lower in T/C-28a2 cells.

In comparison to primary cells, immortalized C-28/I2 chondrocytes bound significantly lower amounts of WGA and *S. tuberosum* lectin (both 1.7-fold), whereas no significant differences were found in case of *D. biflorus* agglutinin and PNA. Of note, remarkable differences were observed for binding levels of *L. culinaris* agglutinin, *S. nigra* agglutinin, and *U. europaeus* isoagglutinin I, which were 3.5-fold lower, 5.7-fold lower, and 11.7-fold higher in case of C-28/I2 chondrocytes, respectively (p<0.05).

Specificity of the lectin–chondrocyte interaction. Competitive assays revealed that lectins were inhibited in their ability to bind to the cell monolayers by increasing amounts of complementary sugars. From these data, IC_{50} values were calculated (Table 2). Due to the low binding of *D. biflorus* agglutinin at the chondrocyte glycocalyx, its binding specificity was only ascertained using excess amounts of *N*-acetylgalactosamine. Using free sialic acid as inhibitory sugar, the preparation of inhibition curves for *S. nigra* agglutinin failed (not shown), whereas sialyllactose as inhibitor markedly inhibited *S. nigra* agglutinin binding to chondrocytes, indicating the necessity of 2,6-linked galactose (Gal) residues for specific *S. nigra* agglutinin binding.

Regarding the specificity of WGA, we found that both *N*-acetylglucosamine (GlcNAc) and triacetylchitotriose induced a high degree of inhibition of the interaction between WGA and the chondrocyte glycocalyx. IC_{50} values resulting from triacetylchitotriose addition, however, were 350 times lower in case of C-28/I2 cells and 220 times lower in

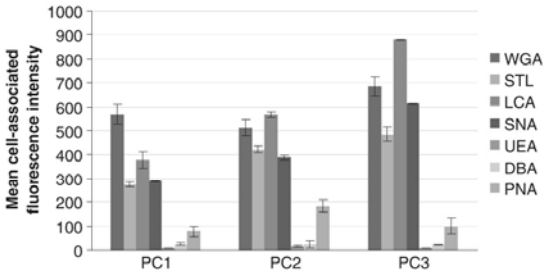


Figure 2. Lectin binding patterns of cultured primary human chondrocytes isolated from three different patients (*PC1–3*). Confluent cell monolayers were incubated with 25 pmol/well fluorescein-labeled WGA, STL, LCA, SNA, UEA, DBA, and PNA for 60 min at 4°C. Results were related to an apparent fluorescein/protein ratio of 1 (mean ± standard deviation, n=4).

Part II: chondrocytes

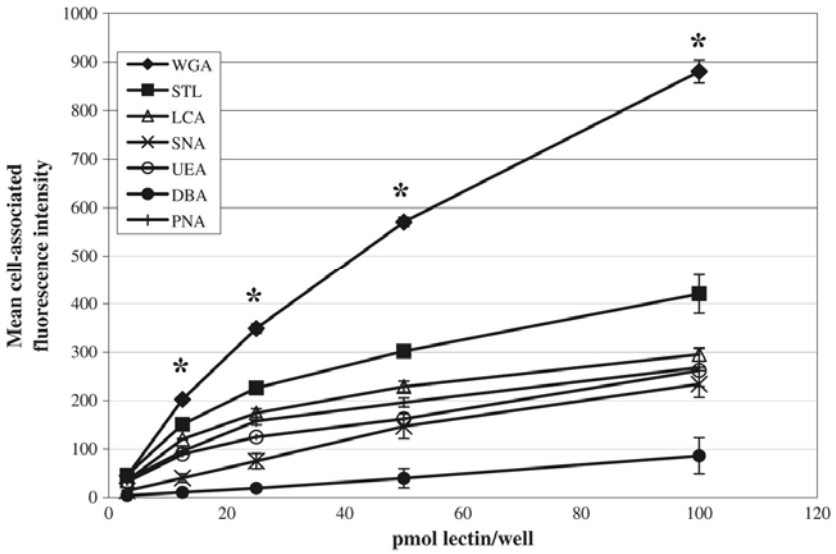


Figure 3. Saturation analysis of lectin binding sites on C-28/I2 chondrocyte monolayers with fluorescein-labeled WGA, STL, LCA, SNA, UEA, DBA, and PNA related to an apparent fluorescein/protein ratio of 1 (mean ± standard deviation, n= 4). Confluent cell monolayers were incubated with 3.125–100 pmol/well fluorescein-labeled lectin in PBS for 60 min at 4°C. The significant differences (p<0.05) between WGA and other lectins at different concentrations are marked with *asterisks*.

case of T/C-28a2 cells as compared to GlcNAc, indicating the preferred binding of WGA to oligosaccharide structures (Table 2). Hence, hyaluronidase-digested hyaluronan consisting mainly of hyaluronan tetrasaccharides was prepared and used as competitive sugar. IC_{50} values observed with these oligosaccharides were 11.6-fold lower in case of C-28/I2 cells and 3.4-fold lower in case of T/C-28a2 cells compared to the monosaccharide GlcNAc. When hyaluronan oligosaccharides were added to *U. europaeus* isoagglutinin I, the low and concentration-independent binding inhibition indicated that hyaluronan oligosaccharides did not exert any unspecific interactions.

For further characterization of WGA and PNA binding specificities, sialic acid residues were enzymatically removed from the chondrocyte glycocalyx. Following neuraminidase treatment, binding of PNA significantly

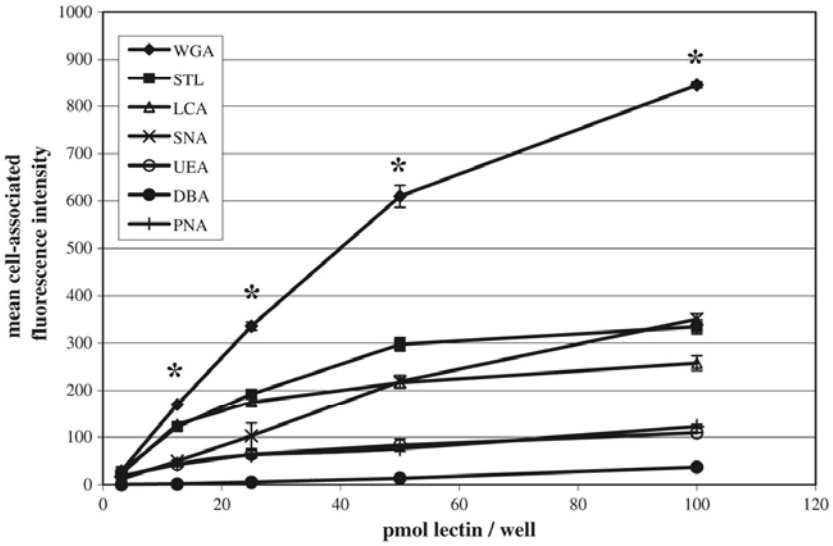


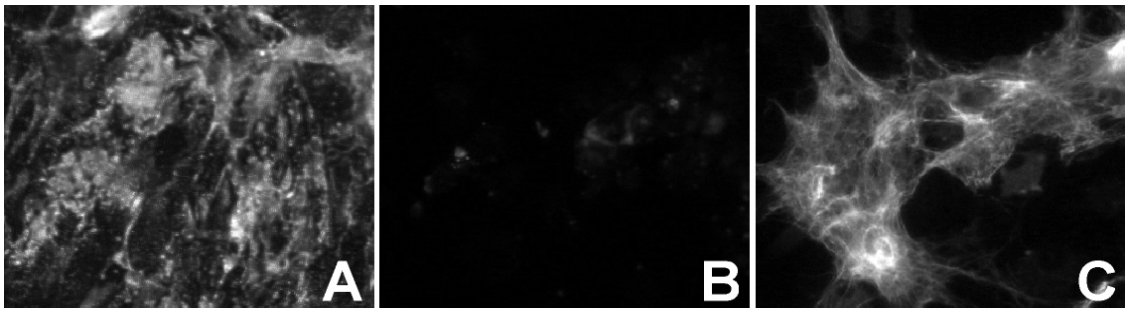
Figure 4. Saturation analysis of lectin binding sites on T/C-28a2 chondrocyte monolayers with fluorescein-labeled WGA, STL, LCA, SNA, UEA, DBA, and PNA related to an apparent fluorescein/protein ratio of 1 (mean ± standard deviation, n=4). Confluent cell monolayers were incubated with 3.125–100 pmol/well fluorescein-labeled lectin in PBS for 60 min at 4°C. The significant differences (p< 0.05) between WGA and other lectins at different concentrations are marked with *asterisks*.

increased (5.3±0.7-fold at 0.05 U/ml neuraminidase) and remained constant upon elevation of neuraminidase concentration. PNA has been shown to specifically bind terminal residues of keratan sulfate which are known to be masked with sialic acid groups (Hoedt-Schmidt et al. 1989). The exposure of these PNA binding sites indicated the successful removal of sialic acids from the chondrocyte glycocalyx and suggested the presence of keratan sulfate. Of note, WGA binding was not significantly affected by neuraminidase treatment (p=0.14 at 0.1 U/ml neuraminidase).

For primary human chondrocytes, competitive assays were performed similarly to the cell lines. Specificity of lectin binding was verified by a continuous decline of fluorescence intensities after addition of increasing amounts of the complementary sugars (data not shown).

Phenotypic characterization of primary and immortalized chondrocytes. In order to associate the evaluated glycophenotype with the general chondrocyte phenotype, the presence of specific chondrocyte markers at C-28/I2 cells and primary OA chondrocytes was assessed using qPCR, immunofluorescence, and GAG analysis. Examination of genes that encode ECM proteins revealed minimal expression of AGC and COL2 in C-28/I2 cells (Fig. 5C), whereas these genes were expressed to a markedly higher extent in primary chondrocytes (13,996 and 32,951-fold, respectively). Regarding COL1, comparable mRNA levels were found in both populations. Of note, COL2 to COL1 ratios, previously defined as a chondrocyte differentiation index (Martin et al. 2001), were markedly higher in the primary cells (53.6 vs. 0.002) and indicated the difference in the molecular phenotype between immortalized and primary chondrocytes. In addition, both cell populations were characterized at the ECM protein level (Fig. 5A). Primary chondrocytes formed a dense ECM including the chondrocyte-specific marker proteins aggrecan and collagen type II, whereas collagen

A)



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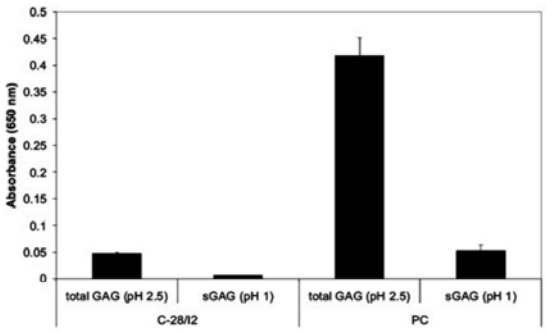

C)

	AGC	COL2	COL1
C-28/I2	0.00004	0.0009	0.46
PC	0.57	31.1	0.58
Ratio	13996	32951	1.27

Figure 5. (*A*) Matrix formation of primary human chondrocytes was evaluated by immunofluorescence staining with primary antibodies for aggrecan (*A*), collagen type I (*B*), and collagen type II (*C*). (*B*) Quantification of alcian-blue stainable proteoglycans present in cultures of immortalized human C-28/I2 chondrocytes and primary human chondrocytes (*PC*). Total glycosaminoglycans (*GAG*) were stained at pH 2.5, whereas sulfated glycosaminoglycans (*sGAG*) were stained at pH 1. Absorbance values were normalized to equal DNA contents as evaluated using the CyQuant assay. (*C*) Quantitative analysis of the mRNA expression of aggrecan (*AGC*), collagen type II (*COL2*), and collagen type I (*COL1*) in immortalized C-28/I2 cells and in primary chondrocytes (*PC*). Expression levels of the PC represent the mean values of three different patients. mRNA levels of each target gene were normalized to GAPDH and calculated as $2^{-\text{deltaCt}}$. For comparison, target gene ratios between PC and C-28/I2 are presented.

Part II: chondrocytes

type I was not present in the ECM of primary chondrocytes. Consistent with the lack of synthesis and secretion of ECM by cells in log-phase growth, immortalized C-28/I2 cells did not stain with either of the antibodies, indicating negligible amounts of ECM accumulated during culture (not shown).

To evaluate the presence of GAG structures in the pericellular coat, C-28/I2 cells and primary OA chondrocytes were stained with alcian blue. At pH 2.5, alcian blue stains most polyanions including polycarboxylic acids such as hyaluronan, whereas at pH 1, only those polyanions that owe their acidity to sulfate groups (e.g., keratin sulfate) are stained. The present experiments revealed alcian blue stainable proteoglycans at the surface of C-28/I2 monolayers both at pHs 2.5 and 1 (Fig. 5B). In comparison to C-28/I2 cells, cultured primary OA chondrocytes revealed significantly higher amounts of alcian blue stainable structures at both pHs 2.5 and 1.

Discussion

In this study, we compared the glycophenotype of primary human chondrocytes and the human chondrocyte cell lines C-28/I2 and T/C-28a2 in relation to their chondrocytic molecular characteristics.

The explicit chondrocytic phenotype of the isolated primary chondrocytes was demonstrated by high expression of ECM genes, ECM proteins, as well as GAG and sGAG structures. The basically chondrocytic phenotype of the C-28/I2 and T/C-28a2 cell lines has been recently demonstrated by detecting SOX9 mRNA levels and marker genes of catabolic activity (Finger et al. 2003). Here, we confirmed the expression of the anabolic marker genes COL2 and AGC in C-28/I2 cells using qPCR assays. As expected for cells in constant log-phase growth, however, the expression of both matrix genes as well as COL2/COL1 ratios were less than in primary chondrocytes in our study. Moreover, alcian blue staining and immunofluorescence confirmed the reduced chondrocytic phenotype of immortalized cell lines as compared to primary cultures.

The present study highlights that differences in the molecular phenotype between immortalized and primary human OA chondrocytes are also reflected in the glycoprofile of the cells. A panel of lectins was selected to probe for a range of oligosaccharide sequences determining specific products of the *O*-glycosylation and *N*-glycosylation pathways (Liener et al. 1986; Baldus et al. 1996; Yang et al. 2004, 2007). In the *O*-glycosylation pathways of most cell types, the primary GalNAc− epitope is converted to the core 1 structure Galβ1-3GalNAc−. These carbohydrate structures may provide binding sites for *D. biflorus* agglutinin and PNA, respectively (Yang et al. 2007). Subsequently, the core 1 structure can be further extended in different ways, e.g., by fucosylation reactions forming the Fucα1-2Galβ1-4GlcNAc− residue which can be specifically detected by *U. europaeus* isoagglutinin I (Baldus et al. 1996). The present experiments show that the C-28/I2 and the T/C-28a2 cell lines differ in their expression of *O*-linked carbohydrates. The binding levels of *D. biflorus* agglutinin, PNA, and *U. europaeus* isoagglutinin I were significantly lower for T/C-28a2 cells, indicating minor biosynthesis of GalNAc− epitopes, core 1 structures, and fucosylation products. The altered glycoprofile of T/C-28a2 cells might be related to their higher proliferation rate as compared to C-28/I2 cells (Goldring et al. 1994; Finger et al. 2003). It has been suggested that the matrix-producing chondrocytic phenotype is negatively correlated with proliferative activity (Finger et al. 2003; Toegel et al. 2008), a fact that might also be reflected in *O*-glycan expression patterns. The average biosynthesis of *O*-glycans in primary human chondrocytes did not significantly differ from that of C-28/I2 cells with respect to the amount of expressed GalNAc− epitopes and core 1 structures. It is remarkable, however, that the expression of Fucα1-2Galβ1-4GlcNAc− residues appeared to be markedly reduced in primary OA chondrocytes.

The biosynthesis of *N*-linked oligosaccharide chains is an intricate process involving the transformation of a high-mannose precursor oligosaccharide to the complex-type oligosaccharide structure by trimming and modification (Helenius and Aebi 2001). To account for *N*-linked oligosaccharide chains, the binding of *L. culinaris* agglutinin and *S. nigra* agglutinin was assessed identifying the expression of high-mannose type oligosaccharides and sialylα2-6Gal termini capping some complex-type *N*-glycans, respectively (Akama et al. 2006; Yang et al 2007). No differences in the expression of these *N*-glycan structures were found between C-28/I2 and T/C-28a2 cells. At primary chondrocytes, the observed susceptibility of *S. nigra* agglutinin and *L. culinaris* agglutinin binding sites to trypsin treatment (Fig. 1) supports the presence of glycoproteins with high-mannose and sialyl structures. Of note, primary OA chondrocytes were characterized by significantly elevated amounts of high-mannose *N*-glycans and sialic acid-capped *N*-glycans in comparison to the cell lines. This observation is in line with previous findings (Bernard et al. 1984) showing that chondrocytes produce mature glycoproteins such as fibronectin containing oligosaccharides of the high mannose or hybrid type in contrast to fibroblast fibronectin, which contains only the complex-type structure. Therefore, we consider the possibility that the reduced levels of high-mannose *N*-glycans in C-28/I2 and T/C-28a2 cells indicate the altered chondrocytic phenotype of immortalized cell lines.

Due to the ubiquitous presence of GlcNAc moieties in the glycocalyx of cells, the target structures of WGA are difficult to specify. First, possible binding sites could be

provided by *N*-linked glycans of the hybrid type. It has been reported that the binding of WGA to the hybrid-type *N*-glycans was mediated by the *N*,*N*′-diacetylchitobiose core portion as well as a GlcNAc residue at the reducing end (Yamamoto et al. 1981). Also, an interaction of WGA with keratan sulfate in cultured human chondrocytes appears conceivable, considering the components of this sGAG. In contrast to *S. tuberosum* lectin, however, WGA has been shown to possess lesser binding affinity for keratan sulfate in its highly sulfated form (Toda et al. 1981). Based on the observation that the binding of WGA to the chondrocyte glycocalyx was higher than that of *S. tuberosum* lectin throughout the study, keratan sulfate might not contribute markedly to the interaction between WGA and cultured chondrocytes. Similarly, the binding of WGA to sialic acid residues in the glycocalyx of cultured chondrocytes appears negligible in view of stable binding values of WGA following enzymatic removal of sialic acids. It has to be underlined that the binding level of WGA was among the highest of all tested lectins with both immortalized and primary chondrocytes. Moreover, the low interindividual variability of WGA binding among different patients might indicate that the WGA binding sites at the glycocalyx are part of the basic repertoire of chondrocytes. Recently, it has been depicted that chondrocytes are surrounded by a several micrometer-thick hyaluronan coat with gel-like properties (Cohen et al. 2003). In our study, alcian blue assays at pH 2.5 suggested high levels of polycarboxylic acids such as hyaluronan covering cultured human chondrocytes. Given the components of hyaluronan, we hypothesize that hyaluronan might represent a target structure for WGA in the pericellular matrix of cultured chondrocytes. The resistance of WGA binding sites against trypsin treatment (Fig. 1) further supports their localization at a nonprotein target such as hyaluronan. In spite of a previous study reporting that WGA does not interact with hyaluronan (Carlsson et al. 1976), our assumption was corroborated by a concentration-dependent inhibition of WGA binding using hyaluronan oligosaccharides. IC_{50} values observed with hyaluronan oligosaccharides were several times higher than those observed with the monosaccharide GlcNAc. Thus, these data suggest the partial binding of WGA to oligosaccharide structures of the chondrocyte hyaluronan coat.

In summary, the present report emphasizes the glycophenotype as an integral part of the chondrocyte phenotype in vitro. In this regard, lectin binding patterns of primary and immortalized chondrocytes indicate that alterations of the chondrocytic molecular phenotype are reflected in the glycophenotype of the cells. In addition to the decreased production of GAGs, the pattern of *O*-linked and *N*-linked oligosaccharides appears shifted toward reduced levels of high-mannose type and sialic acid-capped *N*-glycans as well as increased fucosylated *O*-glycosylation products in immortalized chondrocyte cell lines. A limitation of the current study is that conclusions regarding the glycophenotype of healthy or OA chondrocytes in vivo cannot be definitely drawn from any in vitro model. Based on the presented results, however, future studies might aim to specify the glycoprofile of chondrocytes that is related to OA. This could further clarify the relevance of the glycophenotype for chondrocyte function or its significance, if any, as a marker for the development of new chondroprotective agents.

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Part III: blood-brain barrier

V.E. Plattner, B. Germann, W. Neuhaus, F. Gabor, C.R. Noe, M. Wirth:
Characterization of two blood-brain barrier mimicking cell lines: distribution of lectin binding sites and perspectives for drug delivery.
Submitted to *J Control Release*

W. Neuhaus, B. Germann, **V.E. Plattner**, F. Gabor, M. Wirth, C.R. Noe:
Alteration of the glycocalyx of two blood-brain barrier mimicking cell lines is inducible by glioma conditioned media.
Submitted to *Brain Res,* revised version

Characterization of two blood-brain barrier mimicking cell lines: distribution of lectin binding sites and perspectives for drug delivery

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Part III: blood-brain barrier

ABSTRACT

In the present study plant lectins with distinct sugar specificities were applied to two blood-brain barrier (BBB) mimicking cell lines, namely human ECV304 and porcine brain microvascular endothelial cells (PBMEC/C1-2) in order to elucidate their glycosylation pattern and to evaluate the lectin-cell interaction for lectin-mediated targeting. The bioadhesive properties of fluorescein-labeled lectins were investigated with monolayers as well as single cells using fluorimetry and flow cytometry, followed by confirmation of the specificity of binding. For PBMEC/C1-2 layers highest binding capacity was found for wheat germ agglutinin (WGA), followed by *Dolichus biflorus* agglutinin (DBA) whereas single cell experiments revealed a predominance of DBA only. Analyzing ECV304 monolayers and single cells, WGA yielded the strongest interaction without any changes during cultivation. The binding capacities of the other lectins increased significantly during differentiation. As similar results to primary cells and brain sections were observed, both cell lines seem to be suitable as models for lectin-interaction studies. Thus, an additional focus was set on the mechanisms involved in uptake and intracellular fate of selected lectins. Cytoinvasion studies were performed with WGA for human ECV304 cells and WGA as well as DBA for PBMEC/C1-2 cells. For both lectins, the association rate to the cells was dependent on temperature which indicated cellular uptake.

Keywords: blood-brain barrier; ECV304; PBMEC/C1-2; lectins; drug targeting

INTRODUCTION

Unlike peripheral endothelium, brain microvascular endothelial cells (BMEC) are characterized by minimal pinocytotic activity, the absence of fenestrations and the presence of tight intercellular junctions [1]. They are the main components of the blood-brain barrier (BBB) which maintains the homeostasis of the brain microenvironment and strongly restricts the transport of many drugs from blood to brain.

The surface of the BMECs is covered by a dense layer of complex carbohydrates collectively known as the "glycocalyx" which is thought to contribute to the barrier function [2-4]. Biorecognitive proteins such as lectins are capable of detecting and binding to these carbohydrate moieties at the cell surface by a highly specific interaction. Consequently, lectins may serve as a tool for analysis of the carbohydrate composition of the glycocalyx. Additionally, several studies have outlined the perspectives of lectin-mediated drug delivery [5-7] including glycotargeting of the BBB [8,9].

As investigation of the blood-brain barrier and its function *in vivo* is a challenging task, *in vitro* models mimicking blood-brain barrier characteristics are useful tools for preliminary studies. Primary brain endothelial cells seem to retain more properties of the original tissue, but cultivation is time-consuming and cells have a limited lifespan. Thus, particularly for drug delivery studies cell culture models are preferred as the phenotypic characteristics are stable over a long period including good reproducibility of data. Moreover, cultures of cell lines can easily be cultivated and expanded at will which reduces cost and labor [10]. For that reason, two of the most promising BBB mimicking cell lines, namely human ECV304 and porcine PBMEC/C1-2, were chosen for systematic characterization of the lectin binding pattern at the monolayer and single cell level. The ECV304 cell line was introduced by Takahashi et al. [11] and recommended in a previous study to be suitable for BBB drug transport studies [12]. The cell line PBMEC/C1-2 was established by Teifel and Friedl [13] and used as an *in vitro* model of the BBB in several studies [12,14,15] Both cell lines were cultured in astrocyte conditioned medium (ACM) in order to induce typical BBB properties [12, 16, 17].

The aim of this study was to assess the binding characteristics and binding specificities of several plant lectins with distinct carbohydrate combining sites on these cell lines. To

cover a broad range of sugar structures at the cell surface, lectins were chosen as follows: wheat germ agglutinin (WGA), *Solanum tuberosum* lectin (STL), *Lens culinaris* agglutinin (LCA), *Ulex europaeus* isoagglutinin I (UEA-I), *Dolichus biflorus* agglutinin (DBA) and peanut agglutinin (PNA). Ongoing from these results, an estimation of the glycosylation pattern of cell lines ECV304 and PBMEC/C1-2 and their feasibility as BBB models for elucidation of lectin-mediated drug delivery should be possible. An additional focus was set on the mechanisms involved in uptake and intracellular fate of selected lectins as beneficial for targeted drug delivery.

MATERIALS AND METHODS

Materials

Isocove's modified Dulbecco's medium (IMDM), Ham's F12, Newborn calf serum (NCS), L-Glutamine, Penicillin/Streptomycin and Trypsin/EDTA were bought from Invitrogen Corporation (Gibco^{TM}, CA, USA). Transferrin, Amphotericin B and Gelatine were purchased from Sigma (MO, USA). Heparin was obtained from Fluka (Buchs, Switzerland). Cell culture flasks and 96-well plates were bought from Greiner Bio-one (Kremsmünster, Austria).

Fluorescein-labeled WGA (molar ratio fluorescein/protein (F/P) = 2.9), STL (F/P = 3.1), LCA (F/P = 3.8), UEA-I (F/P = 2.7), DBA (F/P = 2.2) and PNA (F/P=5.2) were purchased from Vector laboratories (Burlingame, USA) and contained >98% active conjugate and no free fluorescein.

Cell culture

Cell line C6 derived from a rat glioma [18] was used in order to produce ACM. They were obtained from the German Cancer Research Center Heidelberg (DKFZ, Heidelberg, Germany) and cultured in C6 medium. The C6 medium is a 1:1 mixture of Ham's F12 and IMDM supplemented with 7.5 % (v/v) new born calf serum (NCS), 7 mM L-Glutamine, 5 µg/ml Transferrin, 0.5 U/ml Heparin, 100 U/ml Penicillin, 100 µg/ml

Streptomycin and 0.25 µg/ml Amphotericin B. In order to obtain ACM the supernatant of a C6 culture was collected every other day.

PBMEC/C1-2 cells were a kind gift from Teifel and Friedl [13] and cultured in PBMEC medium (50 % C6 medium, 50 % ACM).

The ECV304 cell line was purchased from the European Collection of Animal Cell Cultures (ECACC, Wiltshire, UK) and also maintained in PBMEC medium. For single cell studies cells were seeded on gelatine-coated culture flasks and grown to confluence. Cells were harvested by trypsination, centrifuged (1000xg, 4°C, 10 min) and resuspended in PBMEC medium at a concentration of $6x10^6$ cells/ml. For monolayer studies cells were seeded on gelatine-coated 96-well plates at a concentration of 80 000 cells/cm^2 and grown to confluence. Lectin interaction studies were carried out on day 4 with both cell lines and additionally on day 14 with ECV304 cells only. All cells were cultured at 37°C, 5 % CO_2 and 96 % humidity.

Cytoadhesion studies

Lectin binding was investigated using both monolayers and single cells. Monolayers cultivated in 96-well plates were washed twice with 150 µl PBS per well prior to incubation with 50 µl of the particular lectin solution (3.125-100 pmol/well, serial dilution) for 20 min at 4°C. At this temperature the metabolism of the cell is down regulated, and energy dependent transport processes are repressed. Consequently, the amount of cell-associated lectin refers basically to cytoadhesion.

Excess of lectin was removed by washing the monolayers twice with 150 µl PBS. Then, 50 µl PBS were added and the mean fluorescence intensity (MFI) of each well was determined using a fluorescence microplate reader (Infinite M200, Tecan, Grödig/Salzburg, Austria). Blanks were prepared accordingly but using PBS instead of the lectin solution and the values obtained were subtracted from all data quoted. Experiments were carried out in quadruplicate.

Single cells were processed immediately after trypsination. 50 µl cell suspension (3.0 x 10^5 cells) were incubated with 50 µl of a dilution series of the respective lectin in PBS (3.125-100 pmol/assay) for 5 min at 4°C. Then, 100 µl PBS were added and cells were

spun down (1000 rpm, 5 min, 4°C). The supernatant was discarded and this washing step was repeated twice to remove any unbound lectin. To obtain a suitable single cell suspension, the cells were diluted with 1 ml particle free PBS followed by flow cytometric analysis. In order to estimate the autofluorescence of the cells, control samples with unlabeled cells were included in all experiments and the acquired values were subtracted from all data quoted. Experiments were carried out in triplicate.

Specificity of the lectin-cell interaction

Specificity of lectin binding was verified by competitive inhibition using the corresponding carbohydrates (Table 1).
Confluent monolayers were washed twice with 150 µl PBS prior to incubation with 100 µl of the complementary carbohydrate in PBS and 50 µl of the corresponding lectin in PBS (25 pmol/well) for 20 min at 4°C. After a washing step with PBS, cell layers were analysed by fluorimetry after adding 50 µl PBS. For each lectin the amount of inhibitory sugars added is displayed in Table 1.
For single cell experiments, 50 µl cell suspension (3.0 x 10^5 cells), 100 µl of a dilution series of the lectin-specific carbohydrate and 50 µl of a solution containing 25 pmol lectin were processed as described above.

Cytoinvasion studies

In order to investigate potential internalisation of lectins, uptake of WGA by ECV304 cells was studied in detail using single cells. In case of PBMEC/C1-2 cells uptake of WGA and additionally DBA was elucidated.
At this, 50 µl cell suspension (3.0 x 10^5 cells) were incubated with 50 µl of the respective lectin solution (25 pmol WGA or 25 pmol DBA/assay) for 5 min at 4°C (pulse incubation). Unbound lectin was removed as described above, and cells were further incubated for 0-240 min at 37°C or 4°C. As described previously, at 4°C interaction between the lectin and the cell is limited to binding to the cell surface, whereas at 37°C cells are metabolically active allowing energy consuming transport processes. At the end

of the chase-incubation, MFI values were acquired by flow cytometry. Then, 40 µl monensin solution (2.4 mM in EtOH) were added to the cells followed by a further incubation for 3 min at room temperature. Finally, the MFI was assessed again.

Flow Cytometry

Flow cytometric measurements were carried out on an Epics XL-MCL analytical flow cytometer (Coulter, FL, USA). Data were acquired using a forward versus side scatter gate to detect the single cell population and exclude cell aggregates and debris. Fluorescence emission was detected at 525 nm (10 nm bandwidth) after excitation at 488 nm. 3000 cells per sample were acquired and for further calculations the mean channel number of the logarithmic fluorescence intensities of individual peaks (MFI) was used. 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. Data analysis was performed using Coulter System II software 3.0.

Fluorescence microscopy

To confirm binding and uptake of fluorescein-labeled lectins, fluorescence microscopy was used to visualize these processes: ECV304 and PBMEC/C1-2 cells were stained by incubating 50 µl cell suspension (3.0 x 10^5 cells) with 50 µl of the respective lectin solution (25 pmol WGA/assay for ECV304; 25 pmol WGA/assay and 25 pmol DBA/assay for PBMEC/C1-2) for 5 min at 4°C. Then, unbound lectin was removed as described above and cells were further incubated for 240 min at 37°C. Then, cells were washed again and immediately mounted for microscopy without fixation. Control samples were incubated at 4°C. Images of labeled cells were obtained using a Nikon Eclipse 50i microscope equipped with an EXFO X-Cite 120 fluorescence illumination system. Excitation and emission filter blocks were at 465-495/515-55 for green fluorescence. Fluorescence pictures were acquired at 40x magnification using Lucia G v5.0 software for image evaluation.

Statistical analysis

Statistical analyses were carried out comparing two means from independent (unpaired) samples (*t*-test) using the Microsoft Excel® integrated analysis tool. P values of <0.05 were considered as significant.

RESULTS

Lectin-binding capacity of PBMEC/C1-2 cells

Six plant lectins with distinct carbohydrate specificities were selected to estimate the binding capacity of PBMEC/C1-2 cells and thus to characterize the glycosylation pattern of the cell surface. At this, monolayers were grown to confluence and incubated with the fluorescein-labeled lectins. For comparison of the data obtained, MFI values of each lectin were related to an apparent conjugation number of 1 mol fluorescein per mol lectin. Generally, the amount of cell-bound lectin increases with the amount of lectin added. As depicted in Figure 1, the lectin-binding to monolayers follows the order: WGA>DBA>LCA=STL>PNA>UEA-I. In case of WGA and DBA, a pronounced binding was observed in presence of 6.25 pmol lectin and above with fluorescence intensities in the range from 70.0±3.3 to 1476.8±58.8 MFI (WGA) or from 98.3±15.0 to 1097.7±72.6 MFI (DBA).

Additionally, flow cytometric analyses were performed to estimate the binding to single cells. The histograms revealed homogenous staining of all cells without appearance of unstained subpopulations. Interestingly, the resulting saturation curves (data not shown) were different from those of the monolayer assays. The strongest lectin - interaction with the cell surface was observed with DBA (MFI values up to 20.6±1.4) followed by STL>LCA>WGA>PNA>UEA-I. This ranking of lectin-binding capacity remained stable over the applied concentration range between 3.1 and 100 pmol lectin/analysis and is shown in Figure 2 at a concentration of 25 pmol. In this figure, the MFI of the lectin with the highest binding capacity was set to 100% and the binding capacities of the other

lectins were related to this. Thus, a comparison of the lectin binding pattern of monolayers and single cells should be possible, which is also depicted in Figure 2.

Lectin-binding capacity of ECV304 cells

For ECV304 monolayers, which were cultivated for 4 days, highest binding capacity was assessed for WGA with fluorescence intensities between 63.0±7.7 and 1108.1±65.1 MFI (Fig. 3). In presence of 25 pmol WGA/well the fluorescence intensity was 2.9, 5.9, 11.6 or 14.7-fold higher as compared to STL, LCA, UEA-I or PNA, respectively.

In order to follow alterations in the glycosylation pattern during cultivation, saturation experiments were additionally performed with ECV304 monolayers grown for 14 days. Interestingly, after prolonged cultivation WGA binding was only slightly increased with fluorescence values between 100.5±5.7 and 1374.0±43.6 MFI (data not shown), whereas the binding capacity of the other lectins increased notedly. As exemplified at a concentration of 25 pmol, WGA showed no significant increase between 4 days and 14 days cultivation ($p>0.05$), whereas the binding of all other lectins significantly increased ($p<0.01$ for STL, LCA, PNA; $p<0.05$ for UEA-I; Fig. 4). Thus, after 14 days of cultivation, the cell-bound fluorescence intensity of WGA was only 2.2, 2.8, 7.0 and 8.4 fold higher as compared to STL, LCA, UEA-I and PNA.

Analyses of single cells led to saturation curves following the same order as for monolayers (data not shown). WGA yielded fluorescence intensities between 0.4±0.1 and 34.5±2.8 MFI followed by STL (0.0±0.0 to 11.8±1.9 MFI) and LCA (0.1±0.0 to 2.9±0.3). Figure 4 summarizes the lectin-binding capacity at a concentration of 25 pmol/analysis for monolayers and single ECV304 cells. Interestingly, at this concentration LCA possessed a higher binding capacity in comparison to STL (1.4±0.1 MFI as opposed to 0.6±0.2 MFI).

DBA generally yielded very low MFI values scarcely above the autofluorescence of the cells for both, monolayers and single cells.

Part III: blood-brain barrier

Specificity of the lectin-cell interaction

The specificity of the lectin-binding was investigated by competitive inhibition using a dilution series of the corresponding carbohydrate and a constant amount of lectin. Depending on the amount of sugar added, the carbohydrate combining site of the lectin is blocked and inhibited from binding to the cell surface. For confluent PBMEC/C1-2 and ECV304 layers, the applied sugar concentrations and the resulting inhibition values are listed in Table II. These data show that the amount of cell-bound lectin decreases in presence of increasing amounts of the corresponding carbohydrate. Regarding the degree of inhibition, highly specific binding of the respective lectin to cellular structures was determined for ECV304 cells with inhibition values of more than 80% for each individual lectin. In case of PBMEC/C1-2 cells, WGA (93%) and DBA (86%) revealed a highly specific interaction. As the inhibition curves (not shown) are flattening at these percentages, the amount of unspecific binding might be estimated. In case of ECV304 cells, up to 6% of WGA were found to be unspecifically bound, whereas for PBMEC/C1-2 cells the unspecifically bound fraction was at the most about 7% in case of WGA and 14% in case of DBA.

However, it has to be considered that there are major differences in the binding behaviour between lectins and their corresponding mono- and oligosaccharide as well as derivatives due to varying affinities. Thus, the calculated specificities are only valid for the respective sugar used and the quoted specificity is assumed to be an asymptotic approximation commonly used in lectin studies to verify that binding to the cells is specific as it can be blocked by increasing amounts of sugar added.

For comparison of the inhibition data between the cell lines, IC_{50} values were calculated from inhibition curves of each lectin and displayed in Table 2. The IC_{50} is defined as the amount of inhibitory sugar which half-inhibits the lectin-cell interaction.

For WGA and PNA no significant differences were observed between the cell lines, whereas the other lectins revealed a stronger affinity to PBMEC/C1-2 than to ECV304 cells.

Specificity of binding was also ascertained for single cells. For both cell lines, a high degree of specific binding was detected for all lectins (data not shown). Interestingly, the

estimated specificity of UEA-I with PBMEC/C1-2 single cells was higher than that with monolayers yielding an inhibition curve that leveled off at 95% after addition of 0.5 µmol L-fucose. However, this might also be due to a lower affinity of the interaction between UEA-I and the single cells. The other lectins showed similar inhibition values as compared to the monolayer results. As to ECV304 cells, data similar to monolayer assays were obtained for all lectins with highest inhibition values for STL (92% after addition of 0.125 µmol chitotriose).

Uptake of WGA and DBA into PBMEC/C1-2 cells

During pulse incubation at 4°C, WGA was allowed to bind to the cell membrane of PBMEC/C1-2 cells followed by removal of unbound lectin to guarantee identical starting conditions. Then, chase incubation at 37°C was started and resulted in a time-dependent decrease of MFI finally yielding a difference of 3.9±0.3 MFI after 240 min as compared to samples incubated at 4°C for the same time (7.3±0.4 MFI). Upon addition of monensin the reduced MFI could be restored approaching that of the control samples measured immediately after pulse incubation (0 min, 7.0±0.1 MFI). Control samples prepared at 4°C revealed no considerable differences between MFI values prior to and after monensin addition throughout the incubation time (data not shown).

For DBA, the lectin-cell interaction after pulse incubation at 4°C resulted in MFI values of 56.3±6.1. At the end of the chase incubation after 240 min a difference of 33.1±3.6 MFI was detected between samples incubated at 4°C (60.6±4.7 MFI) and 37°C (27.5±5.7 MFI), respectively. In contrast to WGA, the addition of monensin did not result in a full recovery of the reduced fluorescence intensities. It only increased from 27.5±5.7 (240 min, 37°C, without monensin) to 37.1±7.2 (240 min, 37°C, with monensin). Control samples prepared at 4°C reacted as described above (data not shown).

Uptake of WGA into ECV304 cells

In case of ECV304 cells, membrane-association of WGA after pulse incubation at 4°C amounted to 54.0±5.9 MFI. During chase incubation, a difference of 25.8±1.2 MFI was assessed after 240 min between samples incubated at 4°C (53.8±3.7 MFI) and 37°C

(27.9±1.1 MFI). Again, reduced MFI values could be restored upon addition of monensin and control samples prepared at 4°C revealed no differences of MFI values prior to and after monensin addition (Fig. 5).

Fluorescence microscopy

For both cell lines, the images acquired after incubation at 4°C confirmed that the lectins bind exclusively to the surface of the cells as indicated by a fluorescent ring encircling the cell, when the focus was set to the middle of the cells. Incubation at 37°C gave decisive hints towards intracellular enrichment for WGA in case of ECV304 cells and for WGA as well as DBA in case of PBMEC/C1-2 cells, as the fluorescence was predominantly located within the cell (Fig. 6).

DISCUSSION

In this study different plant lectins with distinct sugar specificities were applied to the human cell line ECV304 and the porcine cell line PBMEC/C1-2 in order to elucidate the carbohydrate composition of the glycocalyx and to evaluate the scale of lectin-cell interactions for lectin-mediated targeting. Since precise determination of the total amount of non-specific binding by competitive inhibition assays is sometimes not feasible and both, specific and unspecific binding will contribute to the lectin interaction in vivo when applied for drug delivery purposes, total binding of the lectin was assumed to be most appropriate. Ongoing from the lectin-binding pattern of PBMEC/C1-2 monolayers the detectable carbohydrate composition can be characterized as follows: *N*-acetyl-D-glucosamine and *N*-acetyl-neuraminic acid as corresponding carbohydrates of WGA seem to be prevailing and accessible, followed by *N*-acetyl-D-galactosamine detectable via DBA. *N*-acetyl-D-glucosamine and α-mannose, complementary sugar moieties of STL and LCA, are likewise present, followed by galactosamine (PNA) and L-fucose (UEA-I)-containing structures, which were found to be less abundant. These findings are mostly in accordance to Fischer and Kissel [9] who stained primary capillary endothelial

cells from porcine brain with lectins and detected a strong affinity for WGA, followed by DBA. In this study, no affinity was found for PNA, whereas Plendl et al. showed labeling of endothelial cells isolated from the brain of fetal pigs [19]. Besides WGA, UEA-I was proposed for the characterization of porcine capillary endothelium [9]. Nevertheless, the marginal occurrence of UEA-I binding sites on PBMEC/C1-2 cells was quite predictable as Teifel and Friedl did not detect UEA-I binding of this cell line using a different fixation method and lower concentrations [13]. Previous studies reported alterations in the sugar composition during BBB development [2,4] and a different lectin-binding pattern after changes in the barrier function [20,21]. For PBMEC/C1-2 cells these possible changes in the glyocalyx composition due to the influence of age and differentiation could not be assessed as the cells start to detach within 4 days after reaching confluence [12]. Therefore, lectin binding studies were only carried out on day 4 after seeding.

Experiments with single cells resulted in an apparent predominance of *N*-acetyl-D-galactosamine structures (DBA). Interestingly, *N*-acetyl-D-glucosamine and *N*-acetyl-neuraminic acid residues corresponding to WGA-binding were found to be less abundant whereas the lectin-binding capacities of the other lectins remained similar. These differences might be due to a loss of cellular polarity or the use of trypsin prior to the single cell experiments.

Comparing the results of monolayer and single cell studies, cultivation of PBMEC/C1-2 monolayers appears more appropriate for lectin-interaction studies as the lectin binding pattern on monolayers better correspond to primary endothelial cells and cortical brain sections. Ongoing from this good correlation, this cell line seems to be suitable as an *in vitro* model of the BBB for lectin-mediated drug delivery studies.

Lectin-binding studies using ECV304 monolayers cultivated for 4 days resulted in a glycosylation pattern as follows: *N*-acetyl-D-glucosamine and *N*-acetyl-neuraminic acid moieties as detected by WGA are highly accessible and therefore probably most abundant. According to their detection the other accessible sugar residues follow the order: *N*-acetyl-D-glucosamine (STL)>α-mannose (LCA)>L-fucose (UEA-I)>galactosamine (PNA) and *N*-acetyl-D-galactosamine (DBA). Since ECV304 cells

differentiate over two weeks [12,17], ECV304 monolayers were also cultivated for 14 days. Results revealed significant changes of the amount of the respective lectin-binding sites except for *N*-acetyl-D-glucosamine and *N*-acetyl-neuraminic acid residues reflected by WGA-interaction. Increase of UEA-I binding during cultivation, which was previously found and described as an endothelial cell feature of ECV304 cells [22], underlines once more the endothelial character of this cell line and its utility as an BBB *in vitro* model. No changes were observed with respect to the ranking of the lectin-binding capacities. As above, highest binding rates were detected for WGA.

Single cell assays with ECV304 cells revealed a lectin-binding ranking similar to that of monolayer studies. However, there is only a limited number of accessible binding sites for UEA-I and PNA in comparison to monolayers, so that cultivation of ECV304 monolayers seems more appropriate.

Comparing the monolayer studies after 4 days in culture, both cell lines yielded the highest binding capacity for WGA together with a high binding specificity. The almost complete absence of DBA binding sites on ECV304 cells as opposed to PBMEC/C1-2 cells may be due to the human origin of ECV304 cells as N-acetyl-galactosamine was also scarcely detected in former studies on other human cell lines [23-26]. The binding of the remaining lectins to PBMEC/C1-2 cells was notedly higher than to ECV304 cells.

For lectin-mediated drug-delivery not only binding of the targeter but also uptake is desirable. Ongoing from the characterization of the sugar composition of the two cell lines, WGA and DBA seem to be suitable for further investigations in case of porcine PBMEC/C1-2 cells, whereas only WGA seems to be a promising candidate lectin for human ECV304 cells. For both cell lines internalization of WGA could be demonstrated qualitatively via fluorescence microscopy as exemplified for ECV304 in Fig. 6A and 6B. Ongoing from these results, enrichment of WGA within acidic compartments of the cell was confirmed using monensin. Since the quantum yield of fluorescein is known to be reduced at acidic pH and addition of monensin can compensate the pH-gradient between acidic compartments and the cytoplasm, the decrease of MFI during chase-incubation at 37°C associated with a full recovery of the MFI values after addition of monensin points to lysosomal accumulation of the internalized lectin. As no fluorescent WGA was

detectable in the supernatant, it was even possible to calculate the fraction of WGA taken up into the cell [25]. In case of PBMEC/C1-2 cells, about 30% of initially cell bound lectin were internalized within 10 min, at the end of the chase incubation about 60% were accumulated in acidic compartments. For ECV304 cells, uptake of WGA was almost similar (20% after 10 min and 55% after 240 min). Furthermore, the potential of DBA as targeting vehicle to PBMEC/C1-2 cells could be assessed. Similar to WGA, MFI decreased continuously during chase incubation from 56.3 to 27.5 at 37°C. Interestingly, addition of monensin did not result in total recovery of the initial values. Therefore, possible detachment of initially membrane-bound lectin was considered, but no fluorescent DBA was detectable in the supernatant of the samples. Moreover, lysis of the cell pellets resulted in similar MFI values for both setups (4° and 37°). In addition, fluorescence microscopy also revealed uptake of DBA (Fig. 6C, 6D). Hence, in case of DBA, the quenching effects might result from an accumulation and constriction of the target vehicle in different, not exclusively acidic vesicular compartments of the cell.

All in all, evaluation of the interaction between lectins and the glycocalyx of BBB mimicking cells demonstrated potential applicability of lectins as a tool to improve drug transport across the BBB. For both cell lines, accessible lectin binding sites were detected and the interaction of certain lectins is promising and characterized by high binding strength and specificity. In general, WGA seems to be a suitable candidate for lectin-grafted drug-delivery systems in case of human ECV304 cells, whereas WGA and DBA seem to be promising candidate lectins for porcine PBMEC/C1-2 cells. As similar results to primary cells and brain sections were obtained, both cell lines can serve as models for lectin interaction studies in BBB research, preferentially after cultivation of monolayers.

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TABLES

Table **1**

Specificity of the lectins used in the competitive binding assays

Lectin	MW	Carbohydrate specificity	Inhibitory sugar	Sugar [c] in µmol/analysis (PBMEC/C1-2; ECV304)
WGA	36 000	GlcNAc, NANA	GlcNAc, Chitotriose	0.002-0.063
STL	100 000	GlcNAc	GlcNAc, Chitotriose	0.016-0.25; 0.004-0.125
LCA	49 000	α-Man, α-Glc, α-GlcNAc	D-Man	2-32; 0.5-16
UEA-I	63 000	α-L-Fuc	L-Fuc	0.016-0.5
DBA	120 000	α-D-GalNAc, Gal	GalNAc	0.25-4; n.d.
PNA	110 000	ß-D-Gal-D-GalNAc, ß-D-GalNAc, Gal	D-Gal	0.016-0.5; 0.031-1

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; n.d.: not determined

Table **2**

Competitive inhibition of lectin binding (25 pmol) to confluent PBMEC/C1-2 or ECV304 monolayers by addition of increasing amounts of complementary carbohydrate

	PBMEC/C1-2			ECV304		
Lectin+ Inhibiting sugar	**Sugar [c] in µmol/reaction**	**% inhibition**	**IC_{50}**	**Sugar [c] in µmol/reaction**	**% inhibition**	**IC_{50}**
WGA + Chitotriose	0.002	28.19	0.0036	0.002	27.24	0.0034
	0.004	55.90		0.004	59.70	
	0.008	68.55		0.008	64.32	
	0.016	82.41		0.016	82.42	
	0.031	90.33		0.031	88.89	
	0.063	93.03		0.063	94.55	
STL + Chitotriose	0.016	48.57	0.018	0.004	42.85	0.006
	0.031	65.16		0.008	61.72	
	0.063	77.95		0.016	74.13	
	0.125	82.11		0.031	85.50	
	0.25	83.32		0.063	91.99	
				0.125	96.79	
LCA + D-Man	2	41.54	2.8	0.5	26.73	0.9
	4	63.76		1	56.81	
	8	71.19		2	72.43	
	16	71.22		4	83.95	
	32	82.38		8	90.73	
				16	93.12	
UEA-I + L-Fuc	0.156	0.23	0.18	0.156	27.92	0.08
	0.031	11.16		0.031	29.17	
	0.063	33.79		0.063	43.13	
	0.125	45.26		0.125	68.77	
	0.25	55.89		0.25	80.24	
	0.5	67.57		0.5	87.34	
DBA + GalNAc	0.25	0.69	0.8	-	n.d.	
	0.5	15.32				
	1	73.82				
	2	83.38				
	4	85.53				
PNA + D-Gal	0.156	16.03	0.14	0.031	25.87	0.14
	0.031	25.26		0.063	41.72	
	0.063	39.60		0.125	47.69	
	0.125	48.77		0.25	69.64	
	0.25	58.74		0.5	74.00	
	0.5	78.65		1	80.66	

n.d.: not determined

Part III: blood-brain barrier

FIGURES

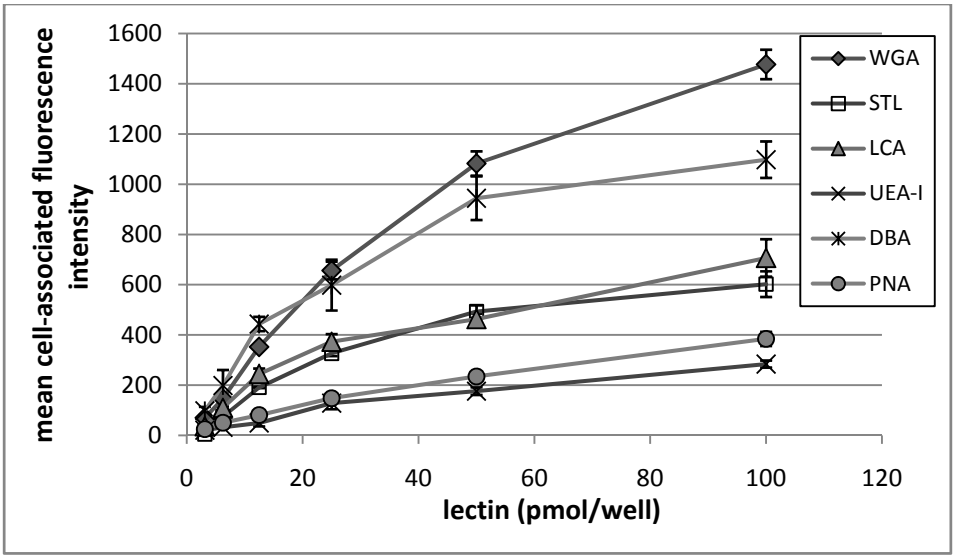

Fig. **1**: Saturation curves of lectin binding to PBMEC/C1-2 monolayers with fluorescein-labeled lectins related to an apparent F/P ratio of 1 (mean±SD, *n*=4).

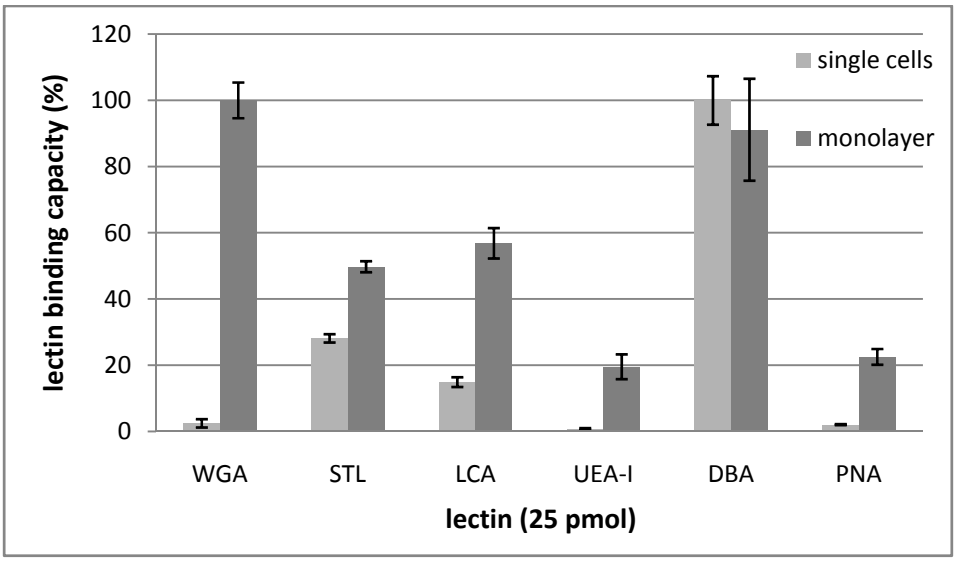

Fig. **2**: Comparison of the lectin-binding capacities of single cells and monolayer of PBMEC/C1-2 cells related to the highest binding capacity using a lectin concentration of 25 pmol (mean±SD, *n*=3).

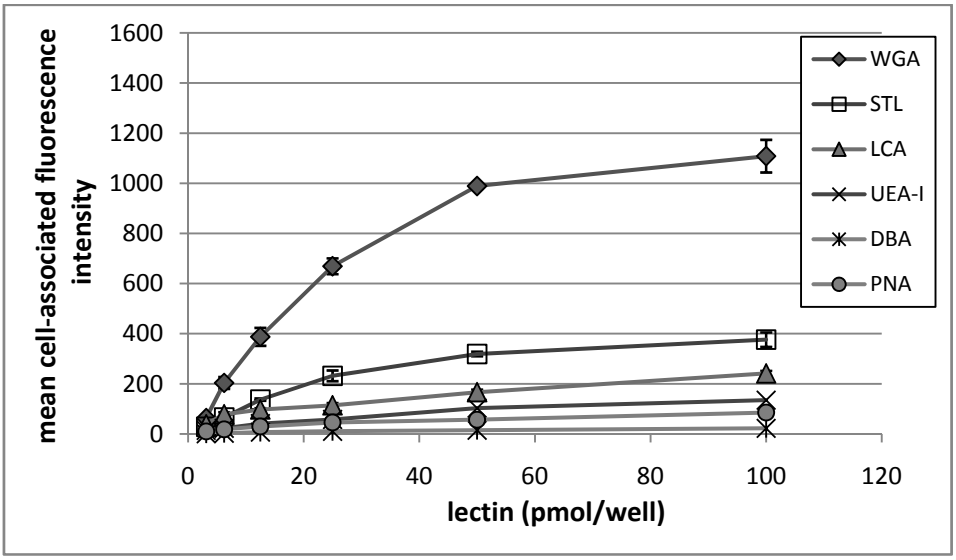

Fig. **3**: Saturation curves of lectin binding to ECV304 monolayers grown for 4 days with fluorescein-labeled lectins related to an apparent F/P ratio of 1 (mean±SD, *n*=4).

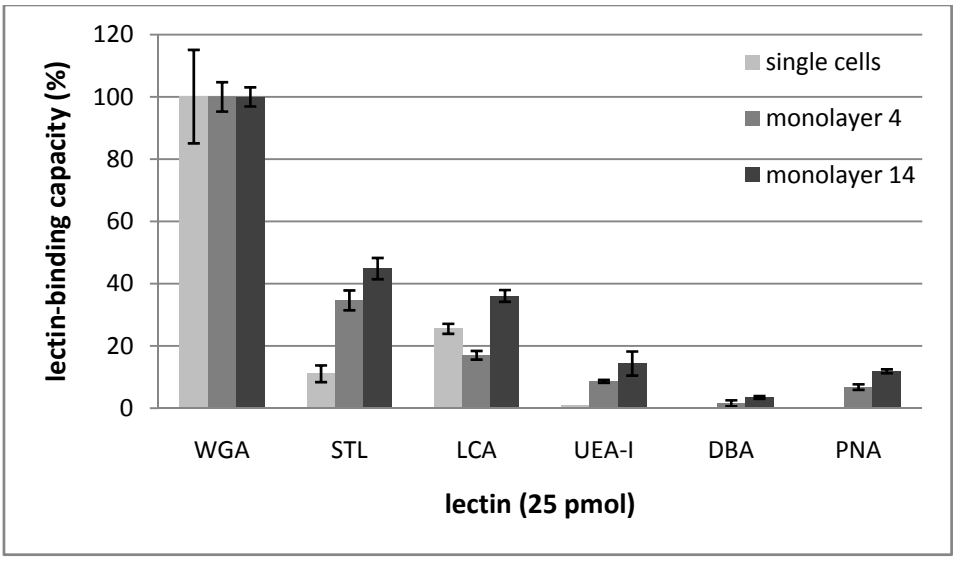

Fig. **4**: Comparison of the lectin-binding capacities of single cells and monolayers of ECV304 cells related to the highest binding capacity using a lectin concentration of 25 pmol (mean±SD, *n*=3).

Part III: blood-brain barrier

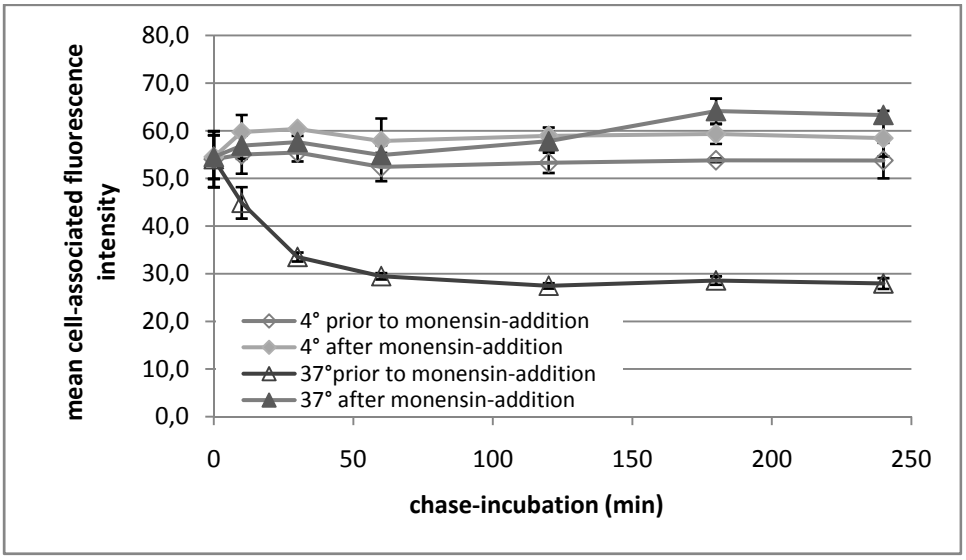

Fig. **5**: Mean fluorescence intensities of ECV304 cells loaded with 25 pmol WGA prior and after addition of monensin during incubation at 4°C and 37°C by time up to 4 h (mean±SD, n=3).

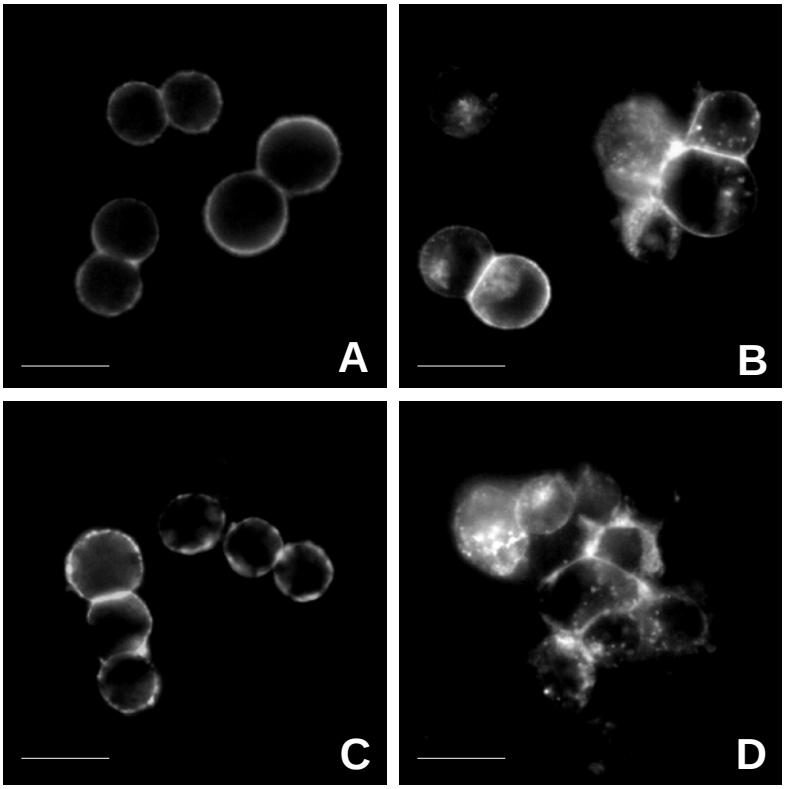

Fig. **6**: Fluorescence microscopy images of ECV304 cells incubated with fluorescein-labeled WGA (25 pmol) for 240 min at 4°C (A) and 37°C (B) and PBMEC/C1-2 cells incubated with fluorescein-labeled DBA (25 pmol) for 240 min at 4°C (C) and 37°C (D). Scale bar represents 20 μm.

Alteration of the glycocalyx of two blood-brain barrier mimicking cell lines is inducible by glioma conditioned media

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Part III: blood-brain barrier

Abstract

Recent studies showed that glioma conditioned medium is able to induce blood-brain barrier properties in brain endothelial cells in vitro. In this regard, it was investigated whether glioma conditioned medium can also influence the lectin-binding capacity of blood-brain barrier in vitro models. For the presented study cell lines PBMEC/C1-2 and ECV304 were chosen because it was previously shown that glioma conditioned medium was able to induce specific blood-brain barrier properties in these cell lines.

Six different plant lectins (WGA, STL, LCA, UEA-I, DBA, PNA) with distinct sugar specificities were applied in order to elucidate the glycosylation patterns of cell line PBMEC/C1-2 and ECV304. Lectin-binding studies were carried out with monolayers as well as with single cells. In the case of PBMEC/C1-2 monolayers, results showed a significant increase of the binding of lectins WGA, STL, UEA-I, DBA and PNA after application of 25 pmol lectin when cultured in media containing soluble factors derived from glioma cell line C6, whereas the binding capacity for LCA remained similar. For ECV304 monolayers, a significant decrease of WGA, STL and LCA was observable, whereas UEA-I binding increased in comparison to cells grown in the corresponding basal growth medium without soluble C6 factors. Single cell studies showed less significant, but similar changes in the lectin-interactions with the cell surfaces.

In conclusion, it was shown that soluble factors derived from glioma cell line C6 can modulate the “glycocalyx” of blood-brain barrier mimicking cell lines.

Section: Regulatory Systems

Key words: blood-brain barrier; lectins; PBMEC/C1-2; ECV304; C6; astrocytes; WGA; DBA; STL; PNA; UEA-I; LCA

1. Introduction

Brain microvascular endothelial cells (BMECs) constitute the main component of the blood-brain barrier (BBB). The BBB maintains the homeostasis of the brain microenvironment which is crucial for neuronal activity and function. Unlike the peripheral endothelium, BMECs are characterized by the presence of tight intercellular junctions, minimal pinocytotic activity and the absence of fenestrations (Joo, 1996). Bands of tight junctions (zonula occludens) between adjacent endothelial cells restrict the paracellular pathway and effectively prevent the passage of polar, hydrophilic drugs through the endothelial cell layer, whereas in non-barrier forming capillaries the bands contain focal discontinuities. The resulting distinct tightness of cerebral capillary junctions is reflected in high transendothelial electrical resistances (TEER) (Butt et al., 1990).

Several recent studies have highlighted the importance of the environmental conditions to induce and maintain BBB properties in brain endothelial cells. Neighbouring astrocytes, pericytes, neurons and even the basal lamina are able to excite BBB properties. In this regard, different components of the extracellular matrix (ECM) as laminin, collagen IV and fibronectin increased barrier properties (Tilling, 1998). Recent experiments showed that the ECM derived from astrocytes and pericytes elevated the electrical resistance of cerebral endothelial cells, whereas ECM from aortic endothelial cells decreased the electrical resistance of the later on cultured cerebral endothelial cells (Hartmann et al. 2007).

In general, the surface of BMECs is covered by a dense layer of complex carbohydrates collectively known as the “glycocalyx”, which is supposed to contribute to the barrier function and can serve as target structure for novel approaches in drug delivery to the BBB. The glycocalyx can be characterized by the use of lectins. Lectins are plant proteins which can selectively bind to specific sugar residues of the cell surface. Stainings with lectins of cerebral endothelial cells in the 1980‘s and early 1990‘s showed that the lectins bound differentially on the luminal or the abluminal side of the BBB (Fatehi et al., 1987; Mann et al, 1992). Furthermore, lectin-staining patterns of BMECs

were changed after different kinds of BBB disrupting incidents. For example, a distinct diminution of the lectin-binding was observed on the luminal side of blood microvessels in cat brains after a cold-lesion injury (Vorbrodt, 1986). On the contrary, the lectin-binding on the abluminal side of the BMEC was altered in the area of an edema. Moreover, translocation of the luminal receptor for Concavalin A to the abluminal side was observed after BBB damage in mice (Vorbrodt et al., 1984). In summary, several studies showed that the lectin-binding changed after alterations of the BBB functionality.
Since it is known that astrocytes, astrocyte conditioned media (ACM), glioma cells and glioma conditioned media (GCM) can induce several BBB properties in endothelial cells as tightness and expression of specific enzymes and receptors (Janzer and Raff, 1987), this study aimed to investigate the possible influence of GCM derived from cell line C6 on the lectin-binding capacity of two BBB mimicking cell lines, namely the porcine cell line PBMEC/C1-2 and the human cell line ECV304.
Cell line PBMEC/C1-2 was introduced by Teifel and Friedl (1996). Typical endothelial markers like von Willebrand factor (vWF) and apolipoprotein A-1 as well as BBB markers like γ-glutamyltransferase (γ-GT) and glucose transporter GLUT-1 were detected in immortalized PBMEC/C1-2 (Teifel and Friedl, 1996). Furthermore, no differences were found in PBMEC/C1-2 between passages 91 and 198 investigating LDL-uptake, expression of vWF, expression of P-gP and activity of γ-GT (Suda et al., 2001). A detailed tightness characterization of PBMEC/C1-2 cultured in different growth media revealed an increase of TEER induced by GCM (Neuhaus et al., 2008a). Moreover, PBMEC/C1-2 were used as BBB model to investigate the invasion of porcine brain microvascular endothelial cells by Streptococcus suis serotype 2 and by Haemophilus parasuis (Vanier et al, 2004 and 2006). Secondly, cell line ECV304 was included in the investigations. Cell line ECV304, which was introduced by Takahashi et al. (1990), exhibits increased TEER when co-cultured with rat C6 glioma cells (Hurst and Fritz, 1996; Easton and Abbott, 2002). This was confirmed by Neuhaus et al. (2008a) using GCM to induce an elevated TEER and an increased expression of tight junctional molecules in ECV304 cells. Furthermore, up-regulation of the glucose transporter GLUT-1 and of gamma glutamyl transpeptidase in astrocyte co-cultured ECV304 cells

were reported (Kuchler-Bopp et al., 1999). In addition, ECV304 developed other BBB characteristics including transferrin receptor expression and P-glycoprotein (P-gP) when co-cultured with rat glioma cell line C6 (Dolman et al., 1998). Moreover, it was shown by Krämer et al. (2002) that co-culture of ECV304 with glioma cell line C6 resulted in a change of the membrane's lipid composition of ECV304 cells to a more *in vivo* like fatty acid pattern. ECV304 cells have also been recommended and used as a BBB in vitro model for drug transport studies (Garberg et al., 2005; Neuhaus et al., 2008a; Neuhaus et al., 2008b).

The aim of this study was to assess the binding characteristics and specificities of several plant lectins with distinct carbohydrate combining facilities on these cell lines and to investigate whether culturing of the cells in GCM results in modified lectin-binding patterns. To cover a broad range of sugar structures at the cell surface, the following lectins were chosen: wheat germ agglutinin (WGA), *Solanum tuberosum* lectin (STL), *Lens culinaris* agglutinin (LCA), *Ulex europaeus* isoagglutinin I (UEA-I), *Dolichus biflorus* agglutinin (DBA) and peanut agglutinin (PNA).

2. Results

Six different lectins were chosen to characterize the glycocalyx of cell lines PBMEC/C1-2 and ECV304 by studying monolayers as well as single cells cultured with and without GCM. In general, the specificity of the lectin-glycocalyx interactions was confirmed for monolayers as well as for single cells of both cell lines with inhibition values of more than 80% for each individual lectin using the corresponding competitive sugars (data not shown).

2.1 Monolayer studies with porcine cell line PBMEC/C1-2

The results of the lectin-concentration dependent binding experiments with PBMEC/C1-2 monolayers grown in basal C6 medium for 4 days are shown in Fig. 1. The lectin-binding to PBMEC/C1-2 monolayers was equally highest for WGA, LCA and DBA

followed by STL, PNA and UEA-I. This order of lectin-binding was constant over the whole concentration range with the exception of WGA that revealed higher values than LCA and DBA using a concentration of 100 pmol/well.

Binding curves were also evaluated for PBMEC/C1-2 cells grown in PBMEC medium (glioma conditioned). Comparing the lectin-binding at 25 pmol to PBMEC/C1-2 monolayers cultured in C6 and PBMEC medium (Fig. 2) significant differences between the two culture media were found. The use of the PBMEC medium significantly enhanced the binding capacity of WGA (185.9 ± 6.6 %), STL (156.1 ± 3.4 %), UEA-I (431.4 ± 19.1 %), DBA (158.7 ± 16.9 %) and PNA (139.5 ± 10.6 %), whereas the binding of LCA to PBMEC/C1-2 remained similar.

2.2 Monolayer studies with human cell line ECV304

The lectin-concentration dependent binding curves of ECV304 monolayers grown in basal ECV304 medium revealed that WGA had the highest binding capacity followed by STL>LCA=UEA-I>PNA>DBA (Fig. 3). The binding of DBA was hardly detectable on ECV304 monolayers. In addition to the comparison of the lectin-binding capacities of ECV304 monolayers cultured in basal ECV304 medium or in GCM of ECV304 medium, the influence of the cultivation time was assessed by investigating lectin-binding patterns of monolayers cultured for 4 or 14 days. The results of all six lectins applied at a concentration of 25 pmol to the monolayers after 4 or 14 days in culture either in basal ECV304 medium or in the GCM of ECV304 medium are summarized in Fig. 4. Generally, most of the lectins bound to a greater extent to the cell monolayers after 14 days culture as compared to a cultivation time of 4 days regardless the growth medium. In particular, binding of WGA, STL, UEA-I, DBA and PNA to ECV304 cells grown in ECV304 medium was also significantly increased just as well the binding of WGA, STL, LCA and PNA to ECV304 cells reared in GCM of ECV304 medium. Results presented in figure 4 also indicate that not only the cultivation period but also the different growth media had an influence on the lectin-binding capacity of ECV304. For ECV304 cells cultured in GCM of ECV304 medium for 4 days the binding of WGA (51.2 ± 10.4 %), STL (75.3 ± 3.7 %) and LCA (68.9 ±13.3 %) was significantly decreased and the

expression of UEA-I (131.2 ± 2.4 %) was significantly increased as compared to ECV304 cells grown in basal medium. The binding of DBA and PNA to ECV304 cells cultured for 4 days was not changed by soluble factors of C6 cells. After cultivation of ECV304 for 14 days in GCM of ECV304 medium, the binding of WGA, STL, DBA and PNA was significantly decreased to 57.5 ± 5.4 % (WGA), 76.9 ± 3.8 % (STL), 26.7 ± 18.5 % (DBA) and 64.5 ± 5.8 % (PNA) as compared to cells cultured in basal ECV304 medium, whereas the binding capacity of LCA and UEA-I remained unchanged.

2.3 Single cell studies

Lectin-binding capacities of the two investigated cell lines PBMEC/C1-2 and ECV304 were also assessed using single cells in order to obtain additional data about the influence of GCM since results can differ dependent on the method used.

Similar to the monolayer studies concentration dependent binding curves were obtained between 3.125 and 100 pmol lectin for both cell lines in the appropriate growth media (data not shown).

At 25 pmol the lectin-binding to PBMEC/C1-2 single cells grown in C6 medium was highest for DBA (8.65 ± 0.46), STL (4.07 ± 0.10) and LCA (2.50 ± 0.26) followed by WGA (0.25 ± 0.09), UEA-I (0.09 ± 0.01) and PNA (0.06 ± 0.01). Culturing PBMEC/C1-2 in PBMEC medium, which contains glioma derived soluble factors, resulted in a similar ranking of the lectin-binding capacities: DBA (13.53 ± 0.99) > STL (3.81 ± 0.17) > LCA (2.02 ± 0.2) > WGA (0.33 ± 0.17) > PNA (0.28 ± 0.02) >UEA-I (0.10 ± 0.04).

Interestingly, the binding of lectins DBA and PNA was significantly increased by culturing PBMEC/C1-2 cells in GCM, whereas the changes of the binding of the other lectins were statistically not significant. Table 2 summarizes the effects of GCM on lectin-binding capacities using monolayers as well as single cells for the analysis. In the case of PBMEC/C1-2 monolayers it was shown that the binding of WGA, STL, UEA-I, DBA and PNA were significantly increased. This was in contrast to the single cell studies where only DBA and PNA bound to a statistically significant higher extent.

Since results of lectin-binding experiments with ECV304 monolayers were quite similar regardless the cultivation duration, ECV304 single cells were only analyzed after 7 days

in culture. The lectin-binding studies at 25 pmol to ECV304 single cells grown in ECV304 medium showed the following ranking: WGA (6.49 ± 0.62) exhibited strongest interactions followed by STL (2.12 ± 0.34), LCA (1.57 ± 0.10) UEA-I (0.09 ± 0.02) and PNA (0.03 ± 0.01). DBA binding was not detectable. GCM of ECV304 medium did not change the ranking but partly the absolute amounts of lectin-binding capacities. WGA interaction decreased to 80 % (5.20 ± 0.75), STL to 92 % (1.96 ± 0.50) and LCA was almost equal at 98 % (1.55 ± 0.02), whereas UEA-I binding increased up to 507 % (0.44 ± 0.06) and PNA to 527 % (0.17 ± 0.01). DBA binding was still not detectable. As indicated in table 2, single cell studies revealed only significant changes in the binding of WGA, UEA-I and PNA, whereas monolayer studies showed different lectin-binding properties of ECV304 cells for WGA, STL, LCA and UEA-I.

3. Discussion

Several studies showed the influence and necessity of astrocytes or their conditioned media for the induction of BBB properties at the BBB or for in vitro BBB models (Janzer and Raff, 1987; Dolman at el., 1998; Kuchler-Bopp et al., 1999). Some further reports revealed a change in the glycocalyx of the BBB resulting in different lectin-binding patterns under BBB disrupting conditions (Vorbrodt, 1986). To our best knowledge, until now no data have been published reporting alterations of lectin-binding patterns of BBB mimicking cell lines with respect to glioma conditioned medium (GCM). Thus, differences of the lectin-binding capacities of the two cell lines PBMEC/C1-2 and ECV304 induced by the usage of GCM were investigated in this study.

The presented lectin-binding studies can be interpreted in conjunction with two aspects. On the one hand, lectins can be used for targeted drug delivery by increasing the uptake of particles or drugs into specific cells by endocytosis after binding to the cellular surface (Fillafer et al., 2008; Weissenboeck et al., 2004; Wirth et al., 1998). Consequently, possible changes of the lectin-binding might be relevant for drug delivery to the brain. On the other hand, because of the specificity of the lectin-binding to distinct sugar

moieties on the cell's surface the lectin-binding patterns describe the compositions of the glycocalyx. This could be relevant for the glycobiology of the BBB in a general manner.

For our studies two BBB mimicking continuous cell lines were selected. One main advantage of cell lines is that they are easier to expand and thus are more accessible for screening and high-throughput purposes, whereas one main disadvantage is the possible loss of key properties in comparison to the *in vivo* BBB situation or even compared to primary cells. We chose two cell lines, which significantly respond to astrocytic signals, namely the porcine PBMEC/C1-2 and the human ECV304. For example, tightness of monolayers of both cell lines increased after growth in GCM in comparison to layers cultured in the corresponding basal media (Neuhaus et al., 2008a). Several studies revealed the suitability of both cell lines for certain BBB studies (Easton and Abbott, 2002; Krämer et al., 2002; Suda et al., 2001; Teifel and Friedl, 1996; Vanier et al, 2004 and 2006). In the case of ECV304 cells, it has to be mentioned that doubt has been raised over the use for BBB studies and whether these cells are endothelial (Brown et al., 2000; Drexler et al., 2002). However, several studies have shown that BBB properties were inducible when ECV304 from ECACC were co-cultured with astrocytes or glioma cell line C6 (Hurst and Fritz, 1996; Easton and Abbott, 2002; Kuchler-Bopp et al., 1999; Dolman et al., 1998). Because of the fact that inducibility of BBB properties is one of the most important features for BBB models, we applied ECV304 cells from ECACC for our studies. Furthermore, ECV304 layers are able to form significantly tighter junctions as reflected in higher TEER values than other BBB cell lines (Tan et al., 2001; Scism et al., 1999; Youdim et al., 2003). Moreover, it was shown that ECV304 were suitable for TEER dependent transport studies (Neuhaus et al., 2008b). In summary, both cell lines possess typical BBB properties making them adequate candidates for the presented studies.

In order to determine the lectin-binding capacities of these two cell lines, studies with monolayers as well as with single cells were accomplished at optimized experimental conditions. Comparison of the PBMEC/C1-2 layers grown either in the basal C6 medium or the PBMEC medium containing GCM revealed significant changes in the lectin-binding capacities. In general, the lectin-binding patterns and rankings of the monolayer

studies were concordant with the data of primary porcine brain endothelial cells or porcine brain sections (Fischer and Kissel, 2001; Plendl et al., 1996).

Using glioma conditioned medium during cultivation, the binding of WGA, STL, UEA-I, DBA and PNA significantly increased, whereas binding of LCA remained similar. These results indicate that the accessibility of almost all corresponding sugar residues was elevated by the influence of glioma secreted soluble factors except for α-mannose (PNA). Furthermore, the 4-5 fold increase of UEA-I binding has to be emphasized which was maybe due to a predominant increase of α-fucose on the cellular surface.

As an additional method lectin-binding studies to single cells were performed. The main advantage of this method using a flow cytometer is the quantitative measurement of the lectin-binding to each single cell. In contrast to the monolayer studies, it was possible to prove that lectins had bound to the single cells uniformly and that it was not a randomly distributed binding only to a part of the total cell collectivity. In general, lectin-interaction studies with monolayers corresponded better to results obtained with primary endothelial cells and cortical brain section than single cell experiments did (Fischer and Kissel, 2001; Plendl et al., 1996). In this context, it has to be mentioned that the absolute numbers of the lectin-binding capacities obtained by the two methods are not to be compared since cells had been treated in different ways before the data acquisition. However, single cell studies revealed no contradicting results to the data of the monolayer tests. DBA and PNA binding increased significantly as compared to the monolayer studies, whereas for all other lectins no significant change was detectable. Long-term studies with PBMEC/C1-2 were not realized since these cells start detaching after 5 days culture at the confluent status (Neuhaus et al., 2008a).

In the case of the human cell line ECV304, binding of WGA, STL and LCA onto the layers was lowered after 4 days cultivation in the GCM as compared to layers grown in the corresponding basal medium, whereas UEA-I significantly increased. Interestingly, UEA-I was reported as a cerebral endothelial marker, which binds to human, rat and porcine brain endothelial cells, but not to goat, dog or mouse (Fatehi et al., 1987; Abbott et al., 1992; Vorbrodt and Trowbrige, 1991; Vorbrodt et al., 1986). Moreover, several hints were published that especially α-fucose, which interacts with UEA-I, plays an

important role during alterations at the BBB (Mann et al., 1992). In concordance with our results that UEA-I binding was elevated by GCM onto monolayers of both cell lines, it is suggested that UEA-I can be used as a distinct marker of the BBB unlike WGA which is capable to bind onto surfaces of various different cell types (Gabor et al., 2001; Plattner et al., 2008; Toegel et al., 2007; Wirth et al., 2002). In the context of drug targeting, WGA enabled the transport of the enzyme horseradish peroxidase into the brain (Banks et al., 1994). However, the usability of UEA-I for lectin-based drug targeting has to be evaluated.

ECV304 layers were also grown for 14 days, since these cells differentiate further with time (Neuhaus et al., 2008a). Comparable to the lectin-binding on ECV304 after 4 days culture, a similar, medium dependent decrease of WGA and STL binding was observed. This might be due to a decrease of *N*-acetyl- D-glucosamine structures. Interestingly, after 14 days of culture the binding of LCA and UEA-I onto ECV304 monolayers was not influenced by GCM, whereas DBA and PNA binding decreased as compared to cells reared in the corresponding basal medium. Besides the qualitative differences of lectin binding onto ECV304 layers with respect to the different growth media, the binding of almost all lectins after 14 days cultivation (except LCA on ECV304 layers reared in ECV304 medium and DBA on ECV304 layers grown in GCM of ECV304 medium) was higher than interactions of the lectins with ECV304 layers after 4 days in culture. This further clearly indicates the influence of the differentiation status of the cell layers on the outcoming results.

Single cell studies with ECV304 after 7 days culture (see table 2) revealed also some medium dependent, significant differences. For example, WGA binding was decreased to 80 %, whereas UEA-I and PNA binding was increased up to 507 and 527 %. Interestingly, the increase of the binding of UEA-I and the decreased WGA interaction was in concordance with the results of the ECV304 monolayer studies.

However, comparing the effects of GCM on the lectin-binding capacities of the two cell lines, it is noticeable that in the case of porcine cell line PBMEC/C1-2 only increases of the lectin-bindings were observed, whereas in the case of human cell line ECV304 most of the lectin-cell interactions decreased compared to cells cultured in basal growth

medium. Considering these different effects the feasibility of the used cell lines for e.g. the development of drug targeting strategies has to be discussed. Generally, the human cell line ECV304 is suggested to be the more predictive model for the human situation. In particular, this was for example supported by the lectin-binding data of DBA. DBA bound to a significantly greater extent to porcine cell line PBMEC/C1-2 than to human ECV304. This was in concordance with other studies, which showed significant DBA binding to porcine primary brain endothelial cells, but negligible interaction with human primary as well as human tumor cells from several tissues (Fischer and Kissel, 2001; Plendl et al., 1996; Plattner et al., 2008; Toegel et al., 2007). Moreover, Mann et al. (1992) investigated the lectin histochemistry of human cerebral microvessels in Alzheimer's disease and concluded that DBA bound only to the basal membrane and not to the endothelial cells themselves. Thus, the weak interaction of DBA with ECV304 cells is one supporting evidence for the superior feasibility of cell line ECV304 in comparison to the porcine cell line PBMEC/C1-2 to mimic the human situation.

However, cell line ECV304 will certainly exhibit also some changed qualities compared to human primary brain endothelial cells. Thus, effects of GCM on the glycocalyx of human primary brain endothelial cells may differ to the presented findings with the cell lines. Furthermore, although it was shown in several reports that glioma cell line C6 induce BBB properties in brain endothelial cells, the influence of primary glial cells may lead to more distinct differences in the lectin-binding patterns than glioma conditioned medium does (Boveri et al., 2005; Smith et al., 2007).

In conclusion, it was shown for the first time that the glycocalyx of BBB mimicking cell lines can be modulated by soluble factors derived from glioma cell line C6 demonstrating that alterations of BBB properties can be induced by the environment, in particular by astrocytes. Until now, it is still unknown whether new and/or additional glycoproteins had been recognized by the lectins or the accessible number of already present sugar structures was increased due to the application of GCM. In this context, correlation of lectin studies with other biochemical techniques is necessary (Fatehi et al., 1987). However, based on the presented data it is suggested that astrocytes or astrocytic soluble factors can modulate the sugar patterns of the glycocalyx of the BBB. This seems quite

important in the context of BBB altering (e.g. during aging, Alzheimer's disease) or damage (e.g. during stroke) and emphasizes the generally growing importance of glycobiology in physiological processes in particular at the BBB.

4. Experimental procedures

4.1 Materials

Amphotericin B, transferrin and gelatin were bought from Sigma (St. Louis, MO, USA), Medium199 (M199), Iscove's modified Dulbecco's medium (IMDM), Ham reg. nutrient mixture F12 (Ham's F-12), newborn calf serum (NCS), foetal calf serum (FCS), L-glutamine and penicillin/streptomycin were obtained from Invitrogen Life technologies (Gibco ^{TM}, Carlsbad, CA, USA). Heparin was purchased from Fluka (Buchs, Switzerland). The fluorescein-labeled lectins and their carbohydrate specificities are listed in Table 1. All lectins were bought from Vector Laboratories (Burlingame, USA) and contained >98 % active conjugate and no free fluorescein. The molar ratio fluorescein/protein (F/P) was 2.9 in the case of wheat germ agglutinin (WGA), 3.1 for *Solanum tuberosum* lectin (STL), 3.8 for *Lens culinaris* agglutinin (LCA), 2.7 for *Ulex europaeus* isoagglutinin I (UEA-I), 2.2 for *Dolichus biflorus* agglutinin (DBA) and 4.7 for peanut agglutinin (PNA). Cell culture flasks and 96-well plates were purchased from Greiner Bio-one (Kremsmünster, Austria).

4.2 Cell culture

Cell line PBMEC/C1-2 was a kind gift from Teifel and Friedl (1996) and was cultured in basal C6 medium and PBMEC medium (50 % C6, 50 % GCM of C6 medium) at passages 61-90. The ECV304 cell line was purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK) and was maintained in basal ECV304 medium and GCM of ECV304 medium at passages 142-164.

The C6 medium is a 1:1 mixture of Ham's F12 and IMDM supplemented with 7.5 % (v/v) NCS, 7 mM L-Glutamine, 5 µg/ml transferrin, 0.5 U/ml heparin, 100 U/ml

penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. The basal ECV304 medium is made of M199 containing 10 % (v/v) FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B. In order to obtain GCM of C6 and GCM of ECV304 medium, rat C6 glioma cells obtained from the German Cancer Research Center (DKFZ, Heidelberg, Germany) were cultured in C6 and ECV304 medium, respectively, and the supernatant was collected every other day (Neuhaus et al., 2008a). All cells were cultured at 37°C, 5 % CO_2 and 96 % humidity.

4.3 Evaluation of the lectin-binding capacity

Lectin-binding capacities were assessed with monolayers as well as with single cells.

For monolayer studies, cells were seeded on gelatin-coated 96-well plates at a concentration of 80 000 cells/cm^2 and grown to confluence. Monolayers were used on day 4 (PBMEC/C1-2, ECV304) and 14 (ECV304 only) after seeding. They were washed twice with 150 µl PBS per well prior to incubation with 50 µl of the respective lectin in PBS (3.125-100 pmol, serial dilution) for 20 min at 4°C. Excessive lectin was removed by washing the monolayers with 150 µL PBS twice. Then, 50 µl PBS were added and the mean fluorescence intensity (MFI) of each well was measured using a fluorescence microplate reader (Infinite M200, Tecan, Grödig/Salzburg, Austria). Negative controls were prepared similarly using PBS instead of lectin solution. Blank studies without cells showed negligible lectin-binding on the gelatin coating.

For single cell studies cells were seeded on gelatin-coated culture flasks and grown to confluence. 4 (PBMEC/C1-2) and 7 (ECV304) days after seeding, cells were harvested by trypsinization, centrifuged (1000 g, 4°C, 10 min) and resuspended in the respective fresh medium. The number of single cells was quantified by microscopy and adjusted to $6x10^6$ cells/ml. 50 µl of the cell suspension were mixed with 50 µl of a solution of the particular lectin in PBS (3.125-100 pmol, serial dilution). In order to estimate the autofluorescence of the cells, control samples with unlabeled cells were included in all experiments. After incubation for 5 min at 4°C cells were collected by centrifugation (1000 g, 4°C, 5 min) and washed twice with 150 µl PBS to remove any unbound lectin. Afterwards the cell suspension was diluted with 1 ml particle free PBS in order to

provide a single cell suspension suitable for flow cytometric analysis. Data acquisition was performed using an Epics XL-MCL analytical flow cytometer (Coulter, USA). 3000 cells per sample were accumulated and the single cell population was gated using the forward versus side scatter signals excluding cell aggregates and debris. Fluorescence emission was detected at 525 nm (10 nm bandwith) after excitation at 488 nm. Amplification of the fluorescence signal was adjusted to put the autofluorescence signal of unlabeled cells in the first decade of the four-decade log range. Data analysis was carried out using Coulter System II software 3.0. For further calculations, the mean channel number of the logarithmic fluorescence intensities of individual peaks (MFI) was used.

As the lectins show different molar fluorescein/protein (F/P) ratios, the results of all experiments expressed as mean cell-bound fluorescence intensities were related to an apparent conjugation number of 1 mol fluorescein per mole lectin to guarantee comparability.

The specificity of the lectin-binding capacities was verified by competitive inhibition with appropriate carbohydrates according to the method reported by Plattner et al. (2008).

4.4 Statistics

All experiments were carried out at least in triplicate. Data are presented as mean ± S.D. Statistics were performed using a two sided student's t-test based on different variances. Probabilities for significant differences are given in the according figure legends.

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Tables

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

Lectin	MW	Carbohydrate specificity	Inhibitory sugar
WGA	36 000	GlcNAc, NANA	GlcNAc, Chitotriose
STL	100 000	GlcNAc	GlcNAc, Chitotriose
LCA	49 000	α-Man, α-Glc, α-GlcNAc	D-Man
UEA-I	63 000	α-L-Fuc	L-Fuc
DBA	120 000	α-D-GalNAc, Gal	GalNAc
PNA	110 000	ß-D-Gal-D-GalNAc, ß-D-GalNAc, Gal	D-Gal

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.

Part III: blood-brain barrier

Table 2: Alteration of lectin-binding capacities of porcine PBMEC/C1-2 and human ECV304 cells grown in the glioma conditioned media as compared to cells cultured in the corresponding basal media.

	WGA	STL	LCA	UEA-I	DBA	PNA
PBMEC/C1-2 cells						
Monolayer, 4 days	+	+	n.s.	++	+	+
single cells	n.s.	n.s.	n.s.	n.s.	+	++
ECV304 cells						
Monolayer, 4 days	-	-	-	+	n.s.	n.s.
single cells	-	n.s.	n.s.	++	n.d.	++

Statistical significances (at least $p < 0.05$) were determined by a two sided student's t-test with different variances. The differences in the lectin-binding were described by following legend: ++ = increase over 200 %, + = increase to 100-200 %, - = decrease to 50-100 %, -- = decrease lower than 50 %; n.s. = not statistically significant, n.d. = not detectable.

Figures

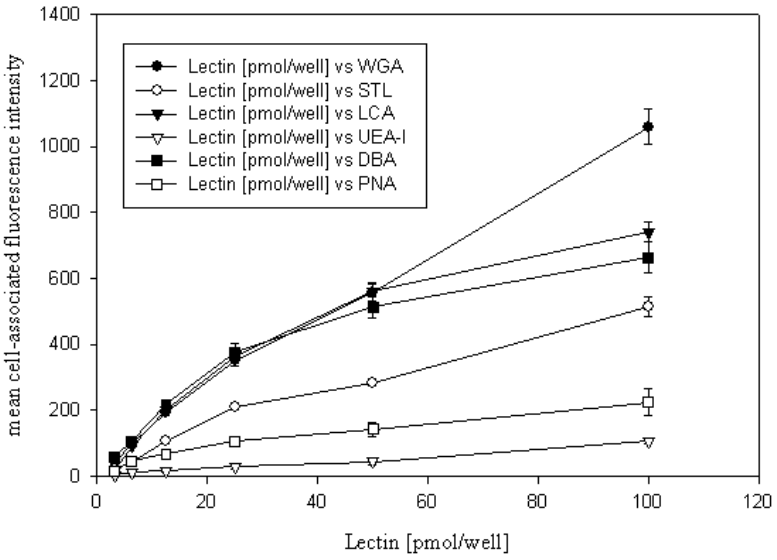

Fig. 1 Concentration dependent binding curves of lectin-binding to PBMEC/C1-2 monolayers cultured in basal C6 medium for 4 days. The fluorescein-labeled lectins were related to an apparent F/P ratio of 1. Data represent mean ± SD (n = 3-4).

Part III: blood-brain barrier

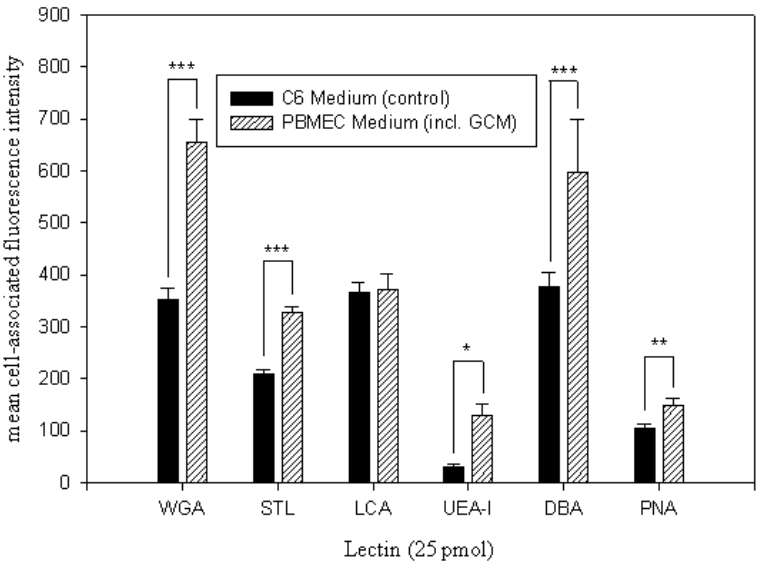

Fig. 2: Comparison of the lectin-binding capacities of PBMEC/C1-2 cells grown in C6 (control) or PBMEC medium (1:1 C6 and GCM of C6 medium) for 4 days using 25 pmol of fluorescein-labeled lectins, incubation: 20 min, 4°C; Data represent mean ± SD, statistical significant differences were determined by student‘s t-test: *=$p<0.05$, **=$p<0.01$, ***=$p<0.005$ (n=3-7).

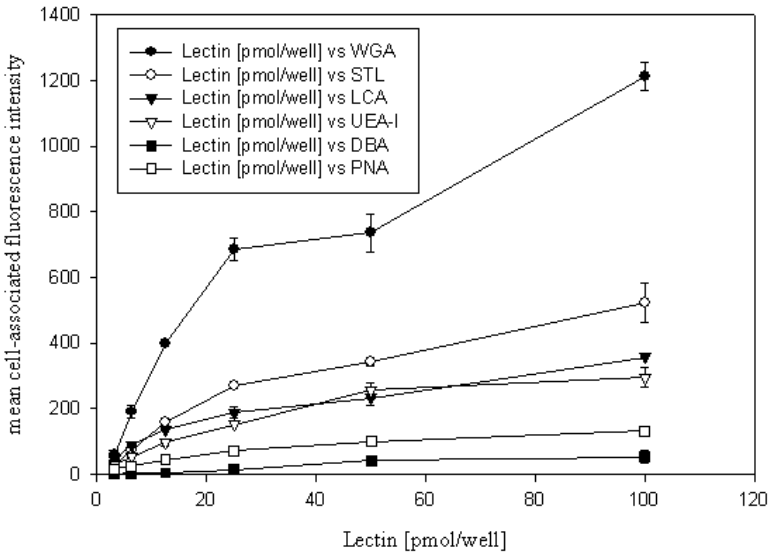

Fig. 3: Concentration dependent binding curves of lectin-binding to ECV304 monolayers cultured in basal ECV304 medium for 4 days. The fluorescein-labeled lectins were related to an apparent F/P ratio of 1. Data represent mean ± SD (n = 3-6).

Part III: blood-brain barrier

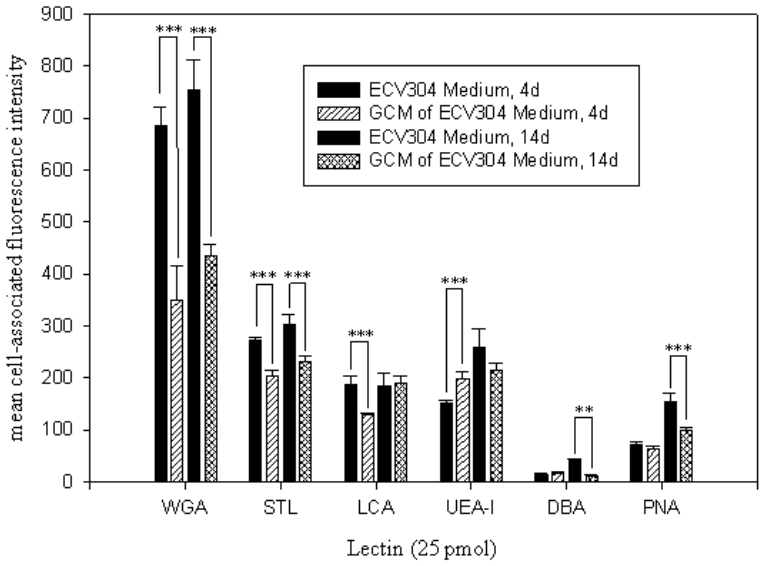

Fig. 4 Comparison of the lectin-binding capacities of ECV304 layers grown in ECV304 or GCM of ECV304 medium for either 4 days or 14 days using 25 pmol of fluorescein-labeled lectins, incubation: 20 min, 4°C; Data represent mean ± SD. Statistical significant differences were determined by student‘s t-test: **=$p<0.01$, ***=$p<0.005$ (n=3-7).

Part IV: monocytes/macrophages

V.E. Plattner, G. Ratzinger, E.T. Engleder, S. Gallauner, F. Gabor, M. Wirth:
Alteration of the glycosylation pattern of monocytic THP-1 cells upon differentiation and its impact on lectin targeting.
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Part IV: monocytes/macrophages

Alteration of the glycosylation pattern of monocytic THP-1 cells upon differentiation and its impact on lectin targeting

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Part IV: monocytes/macrophages

Abstract

In the present study human monocytic THP-1 cells were treated with phorbol 12-myristate 13-acetate (PMA) in order to obtain macrophage-like cells. Before and after treatment, plant lectins with distinct sugar specificities were applied in order to elucidate the glycosylation pattern of both cell types and to follow changes during differentiation.
As a result of flow cytometric analyses, for untreated as well as for differentiated cells wheat germ agglutinin (WGA) yielded the highest binding rate without significant changes in the binding capacity. For the other lectins divergent results were obtained which point to reorganization of sugar residues on the cell surface during differentiation.
Additionally, cytoinvasion being beneficial for targeted drug delivery was studied with WGA which had displayed a high binding capacity together with a high specificity. For both cell types decreased fluorescence intensity at 37°C as compared to 4°C was observable pointing to internalization and accumulation within acidic compartments. Thus, WGA-functionalized PLGA nanoparticles were prepared and their uptake evaluated.
Uptake rates of 55% in case of PMA treated cells suggested that WGA-grafted drug delivery systems might be an interesting approach for treatment of infectious diseases provoked by parasites, facultative intracellular bacteria or viruses such as HIV.

Key words: Monocytes/macrophages; glycosylation pattern; lectins; bioadhesion; drug targeting; nanoparticles

Introduction

Being essential parts of the immune system monocytes and macrophages are involved in defense against microbial infection, initiation of inflammation, immunity to foreign substances, wound healing, and angiogenesis. They originate from the bone marrow and undergo differentiation from hematopoetic stem cells to blood monocytes and finally to tissue-specific macrophages. Like all eukaryontic cells, they are covered by a carbohydrate-rich layer, denominated glycocalyx or pericellular matrix [1, 2]. This cell coat is several tens of nanometers thick and is of functional importance as the first line defense of the innate immune system is relying on recognition of carbohydrates [3]. Furthermore, it plays an important role in cell-cell and cell-matrix interactions. At this, there is evidence that glycocalyx elements influence the endocytic capacity of monocytes [4] and are modulated during adhesion [5,6]. As adhesion of peripheral blood monocytes to endothelial cells initiates the differentiation program of monocytes into phagocytes [7], this process may also cause reorganization of sugar residues at the cell surface.

To investigate the glycosylation pattern of monocytes, which undergo differentiation to macrophages, the human monocytic leukemia cell line THP-1 was chosen, which was established by Tsuchiya et al. and exhibits stable monocytic characteristics [8]. Additionally, treatment with various agents such as DMSO, phorbol 12-myristate 13-acetate (PMA), 1, 25-dihydroxyvitamin D3, retinoic acid or cytokines results in differentiation along the monocytic lineage [9-11]. In summary, it was shown that macrophage-like cells, which mimic native monocyte-derived macrophages in several aspects, are obtained with phorbol esters [12], whereas DMSO or retinoic acid shift the cells rather to characteristics of neutrophils [13].

In the present study, differentiation was carried out with PMA according to Park et al. [14]. In comparison to primary human monocytes, THP-1 cells are characterized by homogeneity, accessibility and a higher transfection efficiency which renders them a reliable cell model.

To identify and characterize the glycocalyx elements of both untreated and differentiated THP-1 cells, binding of fluorescent-labeled lectins was investigated. Lectins represent carbohydrate binding proteins which recognize distinct sugar structures at the cell

surface. In order to cover a broad range of sugar residues at the cell surface several plant lectins with diverse carbohydrate specificities were applied: Wheat germ agglutinin (WGA) and *Solanum tuberosum* lectin (STL) were used to identify *N*-acetyl-D-glucosamine structures and in case of WGA also sialic acid residues. The lectin from *Sambucus nigra* (SNA) specifically recognizes sialic acid (2, 6)Gal/GalNAc sequences, and *Lens culinaris* agglutinin (LCA) binds to mannose residues. Peanut agglutinin (PNA) was used to detect galactosamine moieties, *Dolichus biflorus* agglutinin (DBA) for *N*-acetyl-D-galactosamine-containing structures, and *Ulex europaeus* isoagglutinin I (UEA) specifically binds to fucose.

Ongoing from this characterization and comparison of the pericellular matrices of both cell types, the internalization of WGA was studied in detail. This lectin had already been shown to enhance the phagocytic and bactericidal activity of murine peritoneal macrophages [15], and hence might be a suitable candidate for drug targeting purposes. As site-specific drug delivery promises higher therapeutic efficiency together with a reduction of side effects, there are considerable efforts to identify appropriate targeters which can be used for the functionalization of drug delivery devices. Therefore, nanoparticles were prepared from the biocompatible and biodegradable polymer poly(DL-lactide-co-glycolide) (PLGA) and surface-modified with WGA [16]. Subsequently, their interaction with untreated and differentiated THP-1 cells was studied in order to examine the potential of WGA for targeting nanoparticles to macrophages.

Materials and methods

Chemicals

Fluorescein-labeled lectins from *Triticum vulgare* (wheat germ agglutinin (WGA); molar ratio fluorescein/protein (F/P)=2.9), *Solanum tuberosum* (STL; F/P=3.1), *Sambucus nigra* (SNA, F/P=7.2), *Lens culinaris* (LCA; F/P=3.8), *Arachis hypogaea* (peanut agglutinin (PNA); F/P=5.2), *Dolichos biflorus* (DBA; F/P=2.2) and *Ulex europaeus* (UEA; F/P=2.7) were purchased from Vector laboratories (Burlingame, CA, USA). For the immunofluorescence analysis, FITC-labeled antibodies were obtained from Beckman Coulter (Krefeld, Germany). For particle preparation, poly(DL-lactide-co-glycolide) RESOMER® RG 503 H (PLGA) was purchased from Boehringer Ingelheim (Ingelheim, Germany).

All other chemicals were purchased from Sigma (St. Louis, MO, USA) unless other specified.

Cell culture

Human THP-1 monocytic leukemia cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were seeded at a density of $1x10^5$ cells/ml in RPMI-1640 medium containing 10% heat-inactivated FCS (Biochrom AG, Berlin, Germany), 200 mM L-glutamine and 104 U/ml penicillin, and 104 µg/ml streptomycin and maintained at $3\text{-}8x10^5$ cells/ml in a humidified atmosphere with 5% CO_2 at 37°C.

Differentiation into macrophages was achieved by resuspending the cells at $3x10^5$cells/ml in growth medium supplemented with phorbol-12-myristate-13-acetat (PMA; 8 nM in DMSO) for 48 h. To analyse the surface marker CD14 by immunofluorescence, cells were incubated with PMA also for 24 h and 72 h.

Viability of the cells was determined by trypan blue exculsion and counting the viable cells using a Bürker-Türk hemocytometer. Adherence of differentiated cells was examined with the same setup by determining the amount of unattached and adherent cells. Unattached cells were collected by centrifugation (1000 rpm, 5 min, 4°C) of the supernatant, whereas adherent cells were detached with 0.25% EDTA in PBS followed by centrifugation.

Part IV: monocytes/macrophages

Immunofluorescence analysis of surface antigen CD14

Untreated THP-1 cells were harvested by centrifugation (1000 rpm, 5 min, 4°C) and resuspended in RPMI medium at a concentration of $5x10^6$ cells/ml.

Differentiated cells were detached and collected as described above and re-suspended at the same concentration as untreated cells.

50 µl cell suspension ($5x10^6$ cells/ml) were incubated with 210 µl of the FITC-labeled anti-human CD14 monoclonal antibody solution (MY4-FITC, diluted 1:20 in PBS with 2% FCS) for 30 min at 4°C. After incubation, cells were spun down (1000 rpm, 5 min, 4°C) and the supernatant was discarded. Then, 100 µl PBS were added, and this washing step was repeated to remove any unbound antibody. Finally, cells were re-suspended in 1 ml particle free PBS, and the mean cell-associated fluorescence intensity (MFI) was determined by flow cytometry (Epics XL-MCL, Coulter, FL, USA).

To detect any non-specific antibody binding, additional cells were treated with an adequate isotypic control (MsIgG2b-FITC) or with PBS before analysis.

Lectin binding capacity

Untreated and differentiated cells were harvested as described above and processed immediately. 50 µl cell suspension ($5x10^6$ cells/ml) were incubated with a dilution series of fluorescein-labeled lectins (3.125-100 pmol in PBS, 50 µl) for 15 min at 4°C. Then, cells were washed and prepared for flow cytometry as described above. Negative controls containing PBS instead of the lectin solution were included in all experiments and considered for all calculations. As a control to estimate non specific-binding, samples were prepared as above but using a dilution series of fluorescein-labeled bovine serum albumin (BSA, (F/P=10)) instead of the lectins.

Specificity of the lectin binding

To determine if the binding of the lectins is mediated by carbohydrate moieties at the surface of untreated and differentiated THP-1 cells, 50 µl cell suspension ($5x10^6$ cells/ml), 100 µl of a dilution series of the corresponding complementary carbohydrate and 50 µl of a solution containing 12.5 pmol fluorescein-labeled lectin were incubated

for 15 min at 4°C. The subsequent processing was again performed as described above. Table 1 displays the respective inhibitory carbohydrate for each lectin and the amounts used in the study. According to preliminary results, UEA-I and DBA exhibited no binding to either of the cell lines. Hence, no specificity studies were carried out for these lectins. The specificity of the interaction between SNA and THP-1 cells was not determined as the corresponding carbohydrate sialic acid α2,6 Gal/GalNAc is commercially unavailable.

Internalization of surface bound WGA

To evaluate the internalization of surface bound WGA, 50 µl cell suspension ($5x10^6$ cells/ml) of untreated and differentiated cells were incubated with 50 µl WGA solution containing 12.5 pmol lectin for 15 min at 4°C (pulse incubation). Unbound lectin was removed by centrifugation and washing with 150 µl PBS. Subsequently, cells were further incubated for 0-240 min at either 4°C or 37°C (chase incubation) and then prepared for flow cytometry. After determination of the mean fluorescence intensity (MFI), 40 µl monensin solution (2.4 mM in EtOH) were added and cells were incubated for 3 min at room temperature. Finally, the MFI was assessed again.

Preparation of WGA-functionalized nanoparticles

Nanoparticles were prepared by the solvent evaporation technique. Briefly, 400 mg of PLGA were dissolved in 2 g ethyl acetate. Upon addition of 6 ml 10 % (w/w) aqueous solution of Pluronic® F68, the organic phase was dispersed in the aqueous phase by ultrasonication for 50 s (Bandelin electronic Sonopuls UW 70/HD 70, tip MS 72/D, Berlin, Germany). The resulting O/W emulsion was poured into 100 ml 1 % (w/w) aqueous solution of Pluronic® F68. The organic solvent was allowed to evaporate at room temperature during mechanical stirring (600 rpm) at atmospheric pressure for 1 h and subsequently during 1 h under reduced pressure.

The PLGA nanospheres were covalently coupled with fluorescein-labeled WGA or fluorescein-labeled BSA. For this purpose, 40 ml of the particle suspension were activated by addition of 480 mg N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide

hydrochloride (EDAC) and 20 mg N-Hydroxysuccinimide (NHS), each dissolved in 1 ml 20 mM HEPES/NaOH buffer pH 7.4. After end-over-end incubation for 2 h at room temperature the suspension was washed twice by diafiltration (Vivaflow 50, 100 000 MWCO PES, Sartorius Vivascience GmbH, Goettingen, Germany) using 80 ml of 0.1% Pluronic in 20 mM HEPES/NaOH pH 7.4. The nanoparticle suspension was divided into two equal parts followed by addititon of 20 nmol of WGA (0.72 mg) and BSA (1.32 mg), respectively, each dissolved in 1 ml of the same buffer as above. Following end-over-end incubation overnight at room temperature non-reacted coupling sites were saturated by addition of 300 mg glycine to each tube and further incubation for 1 h. Finally, excess of reagents was removed by three cycles of diafiltration with 40 ml of 0.1% Pluronic in 20 mM isotonic HEPES/NaOH pH 7.4 each. The amount of fluorescein-labeled protein coupled to the particle surface was determined by fluorimetric analysis following dissolution of an aliquot in 0.1M NaOH. The respective nanosphere suspensions contained 3.9 µg/ml WGA and 2.2 µg/ml BSA coupled to the particle surface. The mean particle size as determinded by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments Ltd., UK) was 160.3 nm for WGA-functionalized nanospheres with a polydispersity index (PDI) of 0.130 and 151.2 nm for BSA-coated nanospheres with a PDI of 0.133.

Internalization of surface bound WGA-functionalized PLGA nanoparticles

In order to examine the interaction between WGA- or BSA-coupled PLGA nanospheres and untreated as well as differentiated THP-1 cells, 50 µl cell suspension ($5x10^6$ cells/ml) were incubated with 50 µl nanoparticle suspension for 15 min at 4°C (pulse incubation). Unbound particles were removed as described above, and the cells were further incubated for 30, 60 or 120 min at either 4°C or 37°C (chase incubation). After a first flow-cytometric analysis, 40 µl monensin (2.4 mM in EtOH) were added. Thereupon, cells were incubated for 3 min at room temperature, and the MFI values were determined again.

Flow cytometry

Data were acquired using an Epics XL-MLC analytical flow cytometer (Coulter, FL, USA). In order to exclude any dead cells, debris, and aggregates, viable cells were gated according to their forward versus side scatter properties. From each sample, 3 000 cells were accumulated within this gate. Fluorescence emission of the fluorescence labeled single cell-population was measured at 525 nm (10 nm bandwidth) after excitation at 488 nm. Data analysis was conducted using Coulter System II Software 3.0.

Fluorescence microscopy

Uptake of WGA-functionalized PLGA nanoparticles was visually confirmed by incubating 50 µl cell suspension ($5x10^6$ cells/ml, both untreated and differentiated cells) with 50 µl nanoparticle suspension for 15 min at 4°C (pulse incubation). After removal of unbound particles as described above, the cells were further incubated for 120 min at 37°C. Thereafter, cells were washed again and immediately mounted for microscopy without fixation. Control samples were incubated at 4°C only.

Immunfluorescence images were obtained using a Nikon Eclipse 50i microscope equipped with an EXFO X-Cite 120 fluorescence illumination system. Excitation and emission filter blocks were at 465-495/515-55 for green fluorescence and 510-560/>590 for red fluorescence. Fluorescence images were acquired at 40x magnification and processed using Lucia G v5.0 software for image evaluation.

Statistics

Statistical analyses were carried out using the Microsoft Excel® integrated analysis tool. The hypothesis test among two data sets was made by comparing two means from independent (unpaired) samples (t-test). Values of $p<0.05$ were considered significant. All experiments were performed at least in triplicate.

Part IV: monocytes/macrophages

Results

PMA-induced differentiation of THP-1 monocytes to macrophages

After treatment of THP-1 cells with 8 nM PMA for 48 h, adherence and morphology were examined microscopically. Analysis of cell adherence by counting after trypan blue exclusion revealed that >90% of the cells were attached to the surface after PMA-treatment. Additionally, adherent THP-1 cells were more flattened and elongated indicating the macrophage phenotype as compared to the untreated cells which remained round and in suspension albeit partly aggregated in clusters.

To further ensure differentiation to macrophages, CD14 expression was investigated as the up-regulation of this surface molecule has been reported for maturating monocytes [10,17]. Flow cytometric analysis revealed an increase of CD14 as compared to untreated THP-1 cells. The mean cell-associated fluorescence intensity (MFI) was 0.5 ± 0.3 in case of untreated cells, 0.8 ± 0.0 MFI for cells treated for 24 h, 9.9 ± 0.5 for cells treated for 48h, and 26.0 ± 0.5 MFI in case of cells treated for 72 hours. MFI values of the isotype control were 0.52 in case of untreated cells and 0.57 in case of differentiated cells and subtracted from all data quoted.

Lectin binding capacity of THP-1 cells

Plant lectins with different carbohydrate specificities were used to estimate the glycosylation pattern of untreated and differentiated THP-1 cells. For comparison of the data, MFI values of each lectin were related to an apparent conjugation number of 1 mol fluorescein per mole lectin. For both, untreated and differentiated THP-1 cells WGA yielded the highest binding capacity with MFI values between 1.0 ± 0.0 and 17.1 ± 0.2 (untreated cells) and 0.7 ± <0.1 and 16.6 ± 0.4 (differentiated) respectively (Fig. 1). In case of untreated THP-1 cells, lectin binding was also observed for STL (MFI values up to 10.3 ± 0. 5), PNA (up to 1.0 ± 0.1) and SNA (up to 0.3 ± <0.1) (Fig. 1A). LCA revealed only very low MFI values scarcely above the autofluorescence of the cells (0.1 ± <0.1). For both cell types, binding of UEA-I, DBA as well as BSA, which served as a control for non-specific protein-membrane interactions, were not detectable. Comparison of the two different THP-1 types exhibited for differentiated THP-1 cells also a distinct

interaction with STL (MFI values up to 5.8 ± 0.2) and SNA (1.2 ± <0.1) (Fig. 1B). Interestingly, a notedly higher interaction occurred with LCA (2.4 ± <0.1 MFI at 100 pmol) as compared to untreated THP-1 cells, whereas no binding of PNA was observed. Fig. 2 compares the results at a lectin concentration of 100 pmol. At this concentration, no significant differences ($p>0.05$) were evaluated for WGA, as contrary to all other lectins ($p<0.01$).

Specificity of the lectin-THP-1 interaction

The specificity of the interaction between the plant lectins and THP-1 cells was elucidated by competitive inhibition with complementary carbohydrates (Table 1). For both untreated and differentiated cells, addition of increasing amounts of corresponding carbohydrates resulted in a decrease of the cell-bound lectin. The results are shown exemplarily for differentiated cells in Fig. 3 and indicate the specificity of the lectin-cell interactions. Based on the control sample without any sugar addition representing 100%, the percentage of inhibition was calculated. At this, inhibition values of more than 85% were calculated for WGA, STL, and LCA which points to a highly specific interaction of the respective lectin with cellular structures. In case of untreated cells, similar results were obtained for WGA and STL with inhibition values of about 90% in the same concentration range (data not shown). As LCA had not shown a relevant binding capacity to untreated THP-1, the specificity of the PNA-cell interaction was assessed instead, which also turned out to be specific as represented by more than 90% inhibition at a concentration of 1 µmol galactosamine/reaction (data not shown).

Internalization of surface bound WGA

Fig. 4 depicts the results of the internalization experiments with differentiated THP-1 cells. After pulse incubation at 4°C, the MFI values of surface-bound WGA amounted to 41.5 ± 0.9 at the mean. During chase incubation at 37°C, a time-dependent decrease in fluorescence intensities was observed resulting in 15.8 ± 0.5 MFI after 240 min. In case of undifferentiated THP-1 cells (data not shown), MFI values yielded 40.6 ± 2.0 after pulse incubation and decreased to 14.2 ± 1.1 upon incubation at 37°C for 240 min. After

adding monensin to these WGA pretreated cells, the reduced fluorescence intensity was restored and approached the same level as the control samples at 4°C.
In addition, the same assays were performed for both cell lines also at 4°C where no considerable differences between the MFI values prior and after monensin addition were observable.

Interaction of WGA-functionalized nanoparticles with THP-1 cells

To investigate a potential applicability of lectins for targeting drugs to macrophages the interaction of WGA-functionalized nanoparticles with THP-1 cells was studied. After a pulse incubation at 4°C and removal of unbound particles, the chase incubation was performed up to 120 min at 4°C and 37°C. During chase incubation of untreated cells at 37°C, MFI values decreased from 9.1 ± 0.4 (0 min) to 6.0 ± 0.1 (120 min). Upon addition of monensin, the cell-bound fluorescence intensity was restored reaching 9.1 ± 0.5 (120 + 3 min monensin). In case of differentiated cells, the MFI values declined from 5.7 ± 0.4 (0 min) to 3.2 ± 0.2 (120 min) and re-equilibrated to 5.9 ± 0.2 (120 + 3 min monensin) upon addition of monensin. Chase incubation at 4°C yielded stable MFI values for both untreated and differentiated cells.
In contrast, cell binding of BSA-conjugated nanospheres was not observed. The pulse incubation yielded MFI values of 0.1 ± <0.1 (untreated cells) and 0.2 ± 0.1 (differentiated cells) which are only marginally higher than the autofluorescence of the cells.
Additional information concerning the uptake of WGA-functionalized nanoparticles to untreated and differentiated THP-1 cells was collected via fluorescence microscopy. As illustrated in Fig. 5 for differentiated cells, control samples incubated with WGA at 4°C revealed merely a fluorescent ring of particles around the cells indicating surface binding without any signs of internalization when the focus plane was set to the middle of the cell. In contrast, after incubation at 37°C a dot-like pattern of fluorescence around the nucleus (black spot) was observed pointing to uptake and vesicular enrichment of initially surface bound particles. For untreated cells, similar images were acquired (data not shown).

Discussion

In this study a systematic characterization of the glycosylation pattern of untreated and PMA treated THP-1 cells was performed via detailed analysis of the binding capacities of selected lectins in order to investigate possible changes in course of the differentiation process. Additionally, the potential applicability of the applied lectins for drug delivery purposes at both stages was elucidated. For untreated as well as for differentiated THP-1 cells, the highest binding capacity was found for WGA, a lectin specific for *N*-acetyl-D-glucosamine and sialic acid structures. Regarding the MFI values at 100 pmol, no significant difference ($p>0.05$) between both cell types was detected. STL was the second-best binding lectin for both untreated and differentiated cells, which also recognizes *N*-acetyl-D-glucosamine residues. In contrast to WGA, STL-binding was significantly differed between PMA-pretreated and non-treated cells over the whole concentration range tested. This decrease in *N*-acetyl-D-glucosamine residues might be attributed to an increase of sialic acid moieties present at the cell surface of differentiated cells as indicated by the binding rate of SNA, a lectin which specifically binds to α2,6-linked sialyl groups and yielded a higher binding capacity in case of differentiated THP-1 cells too ($p<0.01$). Significant differences in the lectin binding pattern were also observed for PNA and LCA. In case of untreated cells, galactosamine residues were assessed via PNA, whereas differentiated cells only showed a PNA-binding scarcely above the autofluorescence. In contrast, binding of LCA to mannose structures was very low in case of untreated cells, whereas PMA-treated cells revealed a notedly higher density of mannose at the cell surface. Accessible fucose residues, traceable via UEA, and *N*-acetyl-D-galactosamine moieties, being detectable with DBA, were neither found on monocytes nor on monocyte-derived macrophages.

Sabri et al. [7] demonstrated that increased adhesiveness of stimulated THP-1 cells resulted in decreased cell coat thickness presumably most affecting O-linked carbohydrates. Since Galß1-3-GalNAc represents the core 1 structure of the O-linked chains and is detected by PNA-binding, the loss of PNA-binding capacity upon differentiation of THP-1 cells to macrophages confirms the aforementioned observation Additionally, PMA-differentiation also had an effect on N-linked sugar residues. Among

the motifs on N-linked oligosaccharides, α2, 6-linked sialyl groups detectable by SNA and trimannosyl core groups detectable by LCA are present. For both lectins a significant higher binding capacity was determined on differentiated cells as compared to untreated ones ($p<0.01$) which might be due to upregulation of the CD14 receptor expression during differentiation. CD14 posesses four potential N-linked glycosylation sites with a carbohydrate content contributing to 20% of the total molecular mass [18]. Thus, increased binding of SNA and LCA might occur via these partial structures.

For targeting approaches, not only a high binding rate, but also the specificity of the interaction is an important parameter. Thus, specificity of lectin binding was ascertained yielding inhibition values of at least 85% for each individual lectin.

Uptake of the targeter, a favourable feature in targeted drug delivery, was observed for WGA. For this lectin, the enhancement of the phagocytic and bactericidal activity had been demonstrated with murine macrophages [15]. In our studies, WGA displayed a high binding capacity together with a high specificity for both untreated and differentiated THP-1 cells. In case of a selective approach towards either monocytes or macrophages, LCA might be a suitable candidate, as this lectin exclusively interacted with differentiated THP-1 cells. On the contrary, PNA appears to be useful to target monocytic cells. However, according to a study with murine peritoneal macrophages, this lectin diminished the phagocytic activity suggesting that O-glycosylation is altered during activation of the cells [19]. This observation correlates with our results and thus, the application as a drug targeter is questionable.

The cellular fate of WGA was investigated over a period of 4 h at 4°C and 37°C, respectively. As shown in Fig. 5 the incubation temperature exerted a significant influence on the MFI values: upon chase incubation at 4°C they remained constant indicating an irreversible binding without any evidence for detachment or internalisation of the cell-bound lectin. In contrast, at 37°C the fluidity of the cell membrane increases and active transport processes can occur. Regarding the MFI values of WGA at this temperature, the observed continuous decrease points to internalisation of the lectin and enrichment within acidic compartments, since the fluorescence emission of fluorescein is known to be reduced at acidic pH. Thus, monensin, a carboxylic ionophore which can

compensate the pH-gradient between the cytoplasma and acidic compartments was added to the cells to verify this assumption. After monensin treatment the MFI could be re-established and underlines the obviously pH-dependent and reversible quench of the acid-sensitive label (Fig. 5). As analyses of the supernatants yielded no detached WGA, calculation of the fraction of WGA entering the lysosomal pathway was possible [20]. In case of untreated THP-1 cells, about 15% of the surface bound WGA were accumulated after 10 min, in case of differentiated 25%. The cytoinvasion of WGA at the end of incubation after 240 min amounted to 64% in case of untreated THP-1 cells and 74% in case of differentiated THP-1 cells, respectively. These results indicate that PMA treatment of THP-1 cells might elicit a faster and higher phagocytic activity. Nevertheless, for both cell types a high internalization rate was assessed and thus, WGA seems to be an appropriate candidate for targeting drug carriers to differentiated as well as non differentiated cells.

Moreover, not only uptake of free WGA was observed, but also internalization of WGA-functionalized PLGA nanoparticles of about 160 nm in diameter. Again, incubation at 4°C revealed stable MFI values, whereas incubation at 37°C resulted in a decrease in cell-associated fluorescence intensities by time which could be restored upon addition of monensin. Regarding untreated cells, after 120 min about 40% of the surface bound WGA-grafted particles were internalized and subject to reversible pH quenching. Regarding differentiated cells, at the same time about 55% were taken up into acidic compartments. For comparison, the cell binding of BSA-grafted nanoparticles was assessed, but only marginal cell binding could be observed for both cell types. Cytoinvasion of WGA-functionalized PLGA nanoparticles was additionally confirmed by fluorescence microscopy (Fig. 5). The images acquired after incubation at 37° revealed that the staining of the cytoplasm was not uniformly but in a dot-like manner indicating vesicular accumulation of the particles. Thus, functionalisation of nanocarriers with WGA can lead to enhanced particle uptake into THP-1 cells. This specific interaction might be exploited for lectin-mediated targeting of drug delivery systems to combat infectious diseases evoked by parasites (malaria, leishmaniasis), facultative

intracellular bacteria (brucella, listeria, shigella, mycobacteria) and viruses such as HIV which are known to colonize in the mononuclear phagocyte system [21,22].
In summary, the glycosylation pattern of THP-1 cells showed significant changes due to differentiation with PMA demonstrating a re-organization of sugar residues at the cell surface. Additionally, the endocytic capacity to free WGA and WGA-functionalized nanoparticles was higher in case of PMA treated cells as compared to untreated cells, which might be a result of this rearrangement [4]. In this context, also the potential of WGA as a targeting vehicle was evaluated. Following on from this work, the application of WGA-grafted drug delivery systems might be an interesting approach towards treatment of diseases involving monocytes and macrophages which should be further pursued.

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Tables

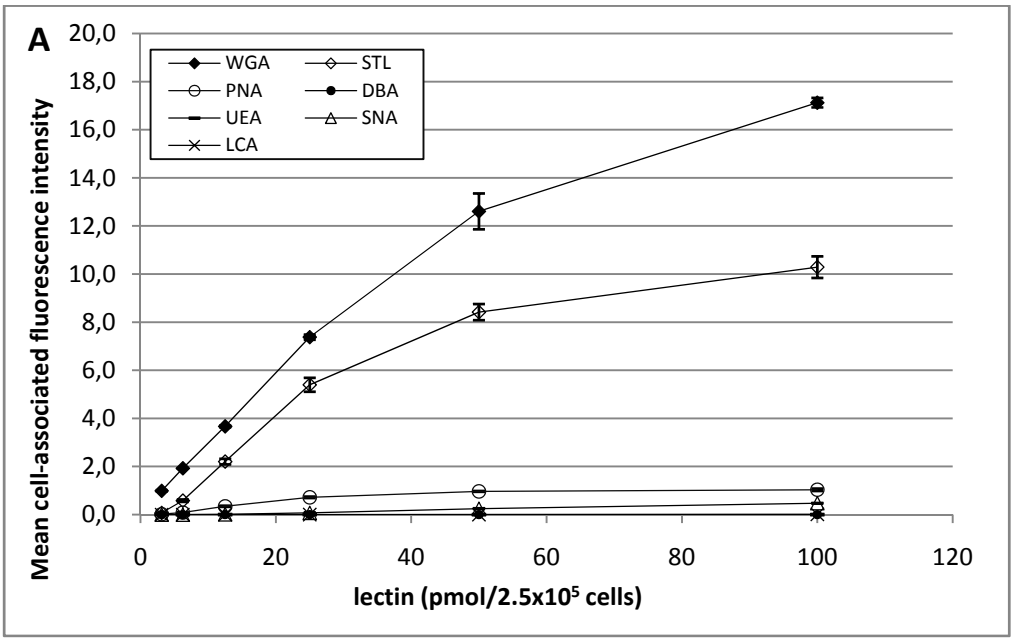
Table **1**

Specificity of the lectins used in the competitive binding assays

Lectin	MW	Carbohydrate specificity	Inhibitory sugar	Sugar [c] in µmol/reaction (untreated/differentiated)
WGA	36 000	GlcNAc, sialic acid	N-acetyl-D-glucosamine, Chitotriose	0.004-0.125/ 0.004-0.125
STL	100 000	GlcNAc	N-acetyl-D-glucosamine, Chitotriose	0.004-0.125/ 0.004-0.125
PNA	110 000	ß-D-Gal-D-GalNAc, ß-D-GalNAc, Gal	D-galactosamine	0.031-1/n.d.
DBA	120 000	α-D-GalNAc, Gal	N-acetyl-D-galactosamine	n.d./n.d.
UEA-I	63 000	α-L-Fuc	L-fucose	n.d./n.d.
SNA	150 000	Sialic acid α(2,6)Gal/GalNAc	-	n.d./n.d.
LCA	49 000	α-Man, α-Glc, α-GlcNAc	D-mannose	n.d./1-32

Part IV: monocytes/macrophages

Figures



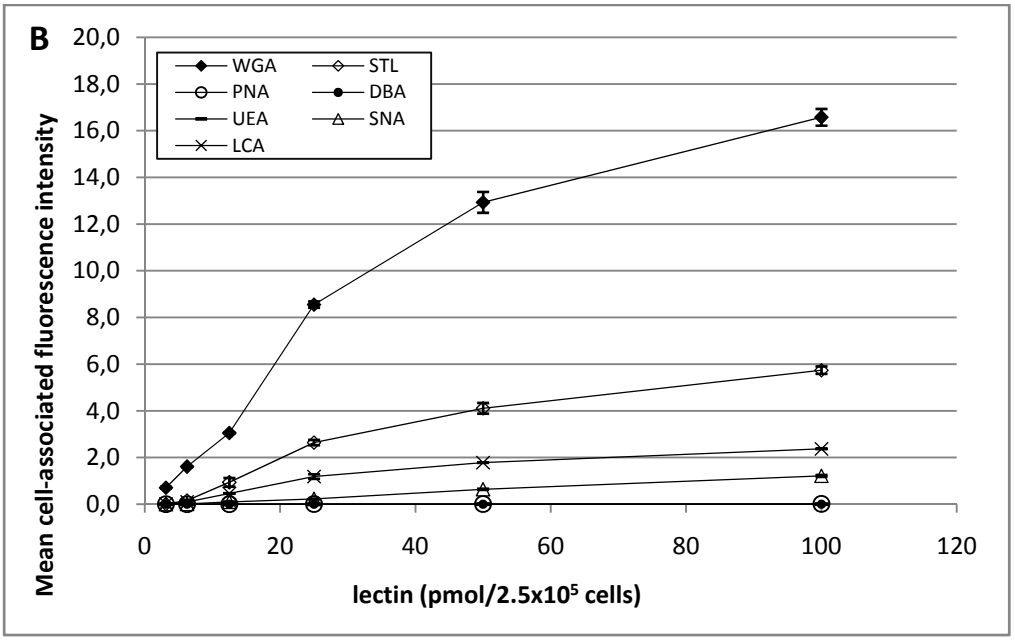


Fig. **1**
Lectin binding to undifferentiated THP-1 (A) and differentiated THP-1 cells (B) at 4°C. The fluorescein-labeled lectins associated with the cell surface were related to an apparent F/P ratio of 1 (mean ± SD, *n*=3).

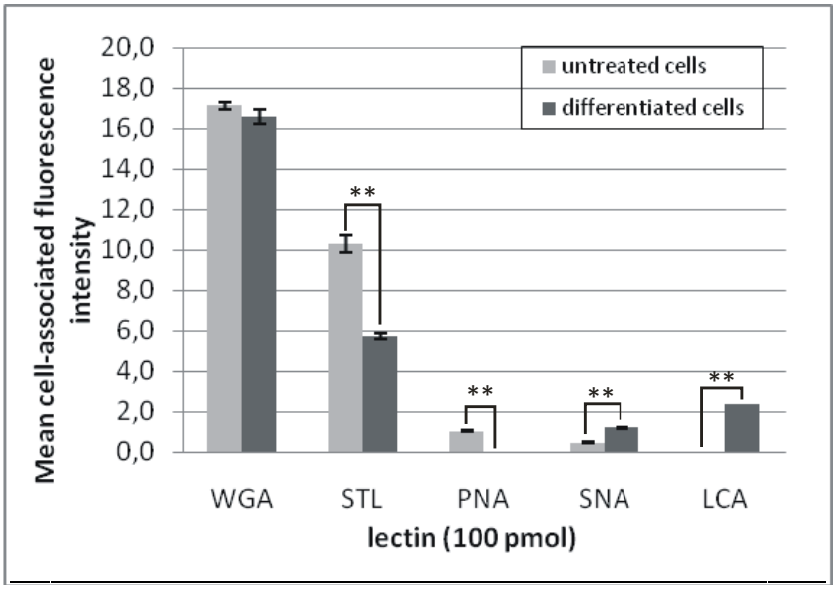


Fig. **2**
Comparison of the lectin-binding capacities of untreated and differentiated THP-1 cells using a lectin concentration of 100 pmol (mean ± SD, *n*=3). Statistical significant differences were determined by student´s t-test: **=p<0.01.

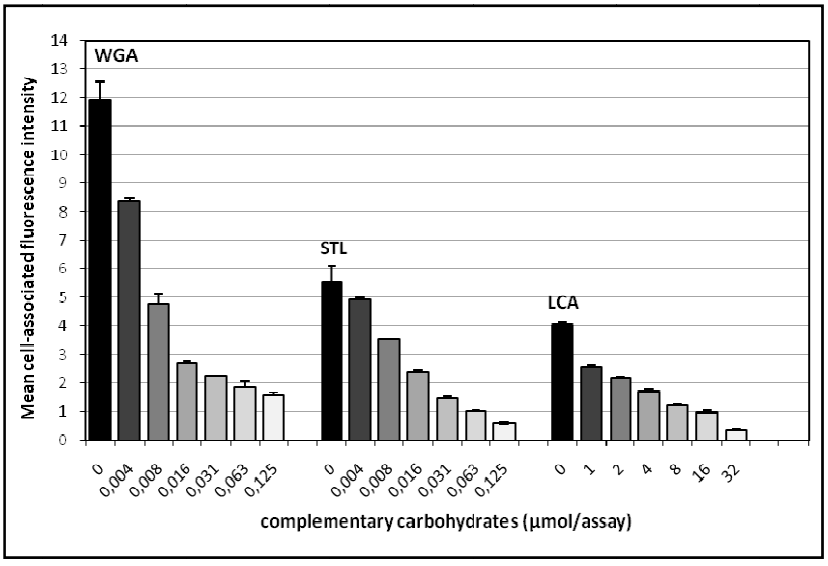


Fig. **3**
Competitive inhibition of lectin binding (WGA, STL and LCA) to differentiated THP-1 cells by addition of increasing amounts of complementary carbohydrate (mean ± SD, *n*=3).

Part IV: monocytes/macrophages

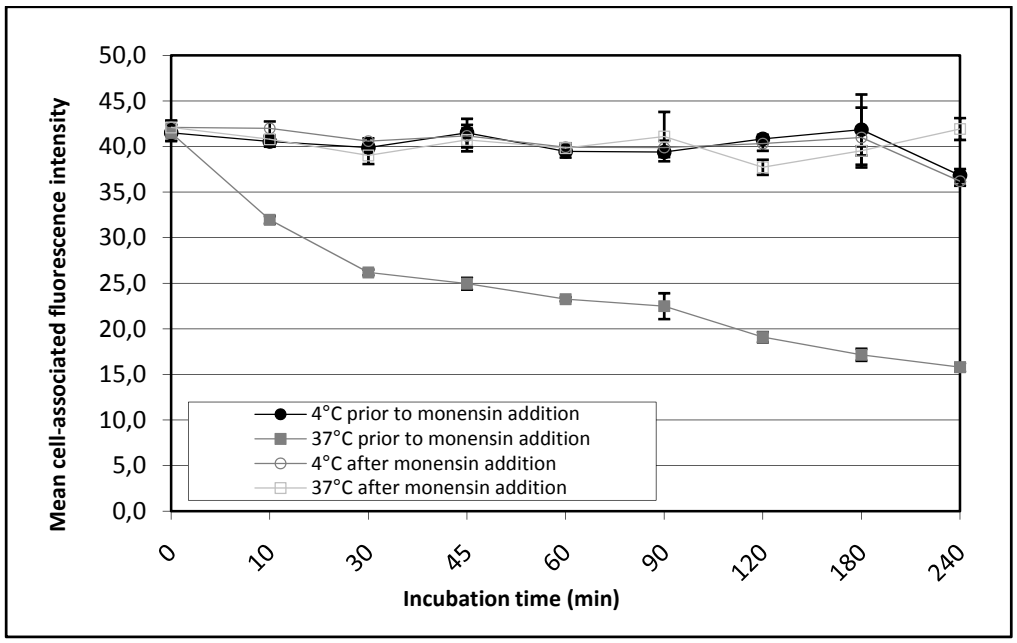


Fig. **4**
Mean cell-associated fluorescence intensities of WGA-loaded differentiated THP-1 cells prior and after monensin addition during incubation (0-240 min) at both 4°C and 37°C (mean ± SD, *n*=3).

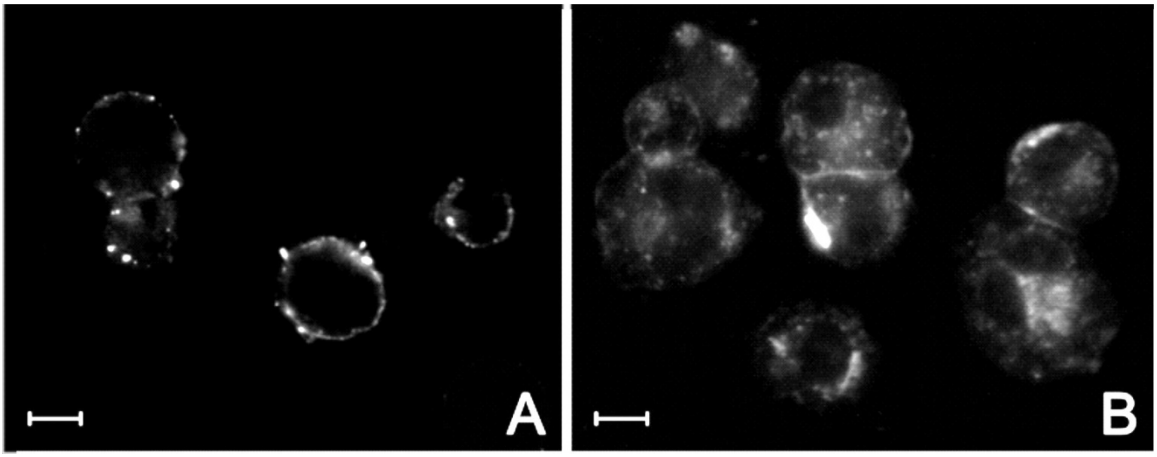


Fig. **5**
Fluorescence microscopy images of differentiated THP-1 cells incubated with WGA-functionalized PLGA nanoparticles for 120 min at 4°C (A) and 37°C (B). Scale bar represents 10 μm

4 Conclusion

The present thesis concentrates on the application of plant lectins as tools for the analysis of the carbohydrate composition of the glycocalyx and the feasibility of lectin-mediated drug delivery for various cell types.

Part I focuses on targeted drug delivery strategies for bladder cancer. In this context, lectins were applied to 5637 cells as a model for human urinary carcinoma (grade II) to estimate the potential and applicability for lectin-mediated targeting. Analyses of single cells as well as monolayers grown for several time intervals pointed to a predominance of *N*-acetyl-D-glucosamine structures accessible via WGA. Thus, the cytoinvasive properties of this lectin were further investigated for bladder tissue. Results revealed accumulation of WGA within acidic compartments and uptake of 50% of the cell-associated lectin after 10 min in case of single cells and after 4 h in case of monolayers. Following on, the cytoadhesive effects of WGA are expected to enhance the therapeutic effect of a cytostatic agent after instillation due to prolonged local action counteracting the loss of drug by urinary excretion.

These improvements are also part of the general motives for a variety of approaches to combat bladder cancer, which were summarized in a review about bladder cancer therapy. In course of this review two main concepts were discussed: therapies with focus on molecular targets ("targeted therapy") and "controlled delivery", a term which was used for all other methods for improved drug delivery. After evaluating the existing strategies, in our opinion, the future is seen in "smart" drug delivery systems such as nanoparticles that contain cytotoxic agents in their core and are decorated with a corona of biologicals which guarantee the specific targeting.

Part II deals with the characterization of the glycosylation pattern of chondrocytes and resulting perspectives for the use of lectins in cartilage research. In the first paper, the affinities of lectins for carbohydrate structures at the surface of human chondrocytes were assessed using single cells of the human chondrocyte cell lines C-28/I2 and T/C-28a2. The results revealed lower lectin binding rates on T/C-28a2 as compared to C-28/I2 which correlates with the observation that T/C-28a2 cells proliferate faster than the C-28/I2 clonal line and therefore show less expression of cartilage matrix components. Nevertheless, for both cell lines, a remarkable cytoadhesion of WGA was observed followed by the conclusion that WGA might be useful for mediating bioadhesion to low-adhesive scaffolds in cartilage tissue engineering approaches. Due to its cytoinvasive properties, which were also assessed in this study, another possible field of application is seen in the preparation of WGA-functionalized drug delivery systems.

As lectins could be successfully administered to C-28/I2 and T/C-28a2 cells, the respective work was the basis for the second paper. This time, the main focus was set on the evaluation of the glycoprofile of primary human chondrocytes and an additional comparison with the two cell lines in order to figure out the contribution of the chondrocyte glycocalyx to matrix assembly and alterations of the chondrocyte phenotype. Using real-time PCR, immunofluorescence, and glycosaminoglycan (GAG) staining, the accurate chondrocytic phenotype of primary chondrocytes was characterized by high expression of extracellular matrix genes and proteins, as well as GAG and sulfated GAG structures, whereas for the immortalized cell lines a reduced chondrocytic phenotype was determined. Analysis of the glycosylation pattern via selected lectins showed reduced levels of high-mannose type and sialic acid-capped *N*-glycans as well as increased fucosylated *O*-glycosylation products for C-28/I2 and T/C-28a2 cells. Thus, alterations of the chondrocytic phenotype were also reflected in the glycophenotype of the cells pointing to a significant role of the latter in chondrocyte differentiation.

In **part III** the elucidation of the lectin-binding pattern of brain microvascular endothelial cells was of particular interest. In the first study, two blood-brain barrier (BBB) mimicking cell lines, namely porcine PBMEC/C1-2 and human ECV304 cells, were cultured in PBMEC medium (50% C6 medium and 50% astrocyte conditioned medium (ACM) of glioma cell line C6), and the bioadhesive properties of selected lectins were assessed using monolayers as well as single cells. Comparison of the results with data from primary endothelial cells and cortical brain sections revealed that PBMEC/C1-2 monolayer cultures appear as an appropriate model for lectin-interaction studies. As to ECV304 cells, the lectin-binding ranking rested stable for single cells as well as monolayers grown for 4 and 14 days. However, due to an increased binding of UEA-I during cultivation, a lectin proposed for the characterization of endothelial cells, the use of ECV304 monolayers seems to be more suitable. To evaluate the impact of lectins for drug targeting purposes, the uptake of WGA and DBA into PBMEC/C1-2 cells was monitored. For ECV304 cells, WGA was estimated to be a suitable targeting vehicle due to its high binding capacity combined with a high binding specificity. For both lectins, the cell association rates were dependent on the temperature indicating cellular uptake.

As lectin-binding has shown to be changed after alterations of the blood-brain barrier functionality, the influence of culture conditions on the lectin-binding pattern of the two cell lines was investigated in the following study. Astrocytes, astrocyte conditioned media (ACM), glioma cells, and glioma conditioned media (GCM) can induce several specific blood-brain barrier properties in endothelial cells including increased tightness and expression of specific enzymes and receptors. Thus, a possible influence of GCM derived from glioma cell line C6 on the lectin-binding capacity was determined. PBMEC/C1-2 cells were cultured in basal C6 medium and PBMEC medium as described above, whereas ECV304 cells were grown in basal ECV304 medium and ACM of ECV304 medium. As significant differences were detected for both cell lines after cultivation in GCM, modulation of the carbohydrate composition on the surface of blood-brain barrier mimicking cell lines by soluble factors derived from C6 cells was

suggested. Ongoing from these findings, the influence of the microenvironment on the glycocalyx of the blood-brain barrier and its role during BBB altering or damage are supposed to be important parameters.

In **part IV** the glycosylation patterns of untreated and differentiated monocytic human THP-1 cells were investigated in order to provide a basis for lectin-mediated targeting to these cell types. Significant changes of the lectin binding capacities before and after treatment of the cells with phorbol 12-myristate 13-acetate (PMA) pointed to a re-organization of the glycoconjugates present at the cell surface during differentiation. However, WGA yielded the highest binding rates for both cell types without significant differences and was therefore chosen for further studies concerning cytoinvasion of soluble WGA. After 4 h high uptake rates of 64% and 74% of initially surface-bound lectin entering the lysosomal pathway were assessed for untreated and differentiated cells, respectively, leading to the preparation of WGA-functionalized PLGA-nanoparticles. Again, high uptake rates were observed, and a comparison with BSA-grafted particles, which did not exhibit feasible binding rates, demonstrated again the potential of WGA as targeting vehicle to monocytes/macrophages due to the specificity of the lectin-cell interaction.

In summary, the present thesis outlines the potential of certain plant lectins to detect and study the carbohydrate composition at the surface of several cell lines. The presented results allow an estimation of the glycosylation pattern of the cell coat and reveal possible alterations due to differentiation, different cell culture types, and media composition. On the basis of the binding assays and continuative studies concerning cytoinvasion, the feasibility of the lectin-mediated targeting concept could be confirmed providing a sound basis for future in-vivo studies.

5 Appendix

German abstract – Kurzfassung

Eukaryotische Zellen besitzen an der Außenseite ihrer Zellmembran einen Kohlenhydratsaum, die sogenannte Glykocalyx, die je nach Zelltyp aus unterschiedlichen Polysacchariden besteht. Neben Proteoglykanen sind diese in der Zellmembran über kovalente Bindung an Membranproteine oder Membranlipide verankert und können von biorekognitiven Proteinen wie Lektinen erkannt werden. Aufgrund dieser Fähigkeiten eignen sich Lektine zur Charakterisierung von Glykosylierungsmustern, die sich im Laufe der Entwicklung und Differenzierung, besonders aber auch im Rahmen einer malignen Transformation der Zellen verändern können. In der vorliegenden Dissertation wurden daher verschiedene Lektine pflanzlichen Ursprungs mit unterschiedlichen Zuckerspezifitäten ausgewählt und deren Interaktion mit folgenden Zelllinien untersucht: humane 5637 Zellen für Studien am Blasenkarzinom, humane C-28/I2, T/C-28a2 und primäre humane Chondrozyten für Knorpeluntersuchungen, porcine PBMEC/C1-2 und humane ECV304 Zellen für die Charakterisierung der Blut-Hirn Schranke und humane THP-1 Zellen als Modell für Monozyten, die die Fähigkeit besitzen zu Makrophagen zu differenzieren. Da einige dieser Zelllinien erst im Zuge der Dissertation in der Arbeitsgruppe etabliert wurden, lag ein Hauptaugenmerk auf einer detaillierten Charakterisierung dieser Zelllinien, um in Zukunft zuverlässige Ergebnisse zu erhalten.

Nach Evaluierung der bioadhäsiven Eigenschaften der eingesetzten Lektine sowie der Charakterisierung der Bindungsspezifität sollte überprüft werden, ob und in welchem Ausmaß Lektine in die Zellen aufgenommen werden. Dieser Aspekt ist bei einem Einsatz von Lektinen als „targeter“ für eine gezielte Pharmakotherapie von großer Bedeutung. In weiterer Folge wurden daher auch Weizenkeimagglutinin (WGA)-funktionalisierte Nanopartikel hergestellt und deren Aufnahme in THP-1 Zellen untersucht, wobei ähnlich wie für WGA alleine hohe Einbauraten erzielt werden konnten.

Zusammenfassend lässt sich festhalten, dass die vorliegenden Ergebnisse eine primäre Bewertung des Glykosylierungsmusters der untersuchten Zelllinien erlauben und die

prinzipielle Eignung eines Lektin-vermittelten Targetingkonzeptes aufzeigen, sodass diese Arbeit eine Grundlage für weiterführende in vivo-Studien darstellt.

Curriculum vitae

Personal data

Name	Verena Elisabeth Plattner, maiden name Söllner
Date of birth	23/02/1981, Vienna, Austria
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Education

since 09/2005	PhD studies in natural sciences (pharmacy) at the University of Vienna
09/2004-09/2005	Completion of the pharmacist education (Aspirant) in "Vindobona" pharmacy, Vienna September 2005: very successful graduation (license as a qualified pharmacist in Austria and Europe)
10/1999-06/2004	Studies in pharmacy at the University of Vienna 2003 and 2004 scholarships for academic excellence June 2004: Mag. pharm. (MPharmSC) degree awarded
09/1991-06/1999	Secondary school (Gymnasium) with emphasis on languages, Wasagymnasium, 1090 Vienna June 1999: very successful graduation

Professional experience

since 09/2005	University assistant in training at the Department of Pharmaceutical Technology and Biopharmaceutics, University of Vienna, Associate lecturer in the practical course on magistral formulae
02/2008	Lab supervisor of the module "Cellular binding and uptake" on the 7th workshop on biological barriers and nanomedicine, Saarland University, Saarbrücken
08/2006-01/2008	Teaching assignment for pharmacology at a school for nurses for the elderly, "Soziales Netzwerk", 1030 Vienna
09/2004-09/2005	Professional experience in "Vindobona" pharmacy, 1090 Vienna
2003-2004	Project collaborator and diploma student at the Department of Clinical Pharmacology, Medical University of Vienna
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