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DIPLOMARBEIT

„Charakterisierung des Glykosylierungsmusters der Mittelohrschleimhaut von Meerschweinchen“

verfasst von

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Inhaltsverzeichnis

1. Einleitung und Problemstellung

Otitis media (OM) ist eine der häufigsten entzündlichen Erkrankungen im Säuglings- und Kleinkindalter und somit oftmals Grund für eine Antibiotika-Therapie in der Pädiatrie [1]. Die herkömmliche Therapie führt jedoch auf Grund mangelnder Penetration des Wirkstoffes in die Mittelohrschleimhaut (MEM - middle ear mucosa) [2], eingeschränkter Zugänglichkeit zu den Bakterien durch Bildung eines Biofilms [3], sowie der geringen symptomatischen Besserung innerhalb der ersten 24 Stunden [4], oft nicht zum gewünschten Behandlungserfolg. Insbesondere die Behandlung von Kindern mit OM erfordert neue Therapieansätze, die eine rasche Linderung der Symptome und damit eine höhere Compliance der jungen Patienten ergeben. Um durch neuartige Applikationsformen den therapeutischen Erfolg zu steigern und die Wirkstofffreisetzung zu optimieren, wurden thermoreversible Formulierungen [5,6], Ohrentropfen [7], Inserts [8], Mikropumpen [9], Hydrogele [10], Wirkstoff-haltige Mittelohrprothesen [11,12,13] oder Pellets [14] zur teilweise lokalen Anwendung bei OM entwickelt. Eine lokale Therapie im Mittelohr wird allerdings durch physiologische Gegebenheiten limitiert. Die mit der Paukenhöhle verbundene Eustachische Röhre kann zu einem Wirkstoffverlust durch Drainage von intratympanal applizierten Lösungen oder Suspensionen führen. Zusätzlich kommt es durch diesen Abfluss nur zu einem kurzen Kontakt des Wirkstoffes mit der MEM und damit auch den Bakterien, sodass ein geringer therapeutischer Effekt hervorgerufen wird. Aus diesen Gründen ist es von Vorteil, den Wirkstoff an der MEM zu verankern, um dadurch einerseits die Verweildauer des API in der Paukenhöhle zu verlängern und andererseits den Wirkstoffverlust durch die Eustachische Röhre zu verhindern. Diese Strategie könnte durch Entwicklung von bioadhäsiven Trägersystemen, die mit der MEM interagieren für die OM-Behandlung umgesetzt werden [15]. Um dieses Konzept zu verwirklichen können pflanzliche Lektine eingesetzt werden, die spezifisch an bestimmte Zuckerstrukturen der Zelloberfläche binden. Dieses Bioadhäsionskonzept von Lektin-vermitteltem Targeting wurde bereits in-vitro für diverse biologische Barrieren [16] wie Urothel [17,18], Blut-Hirn-Schranke [19], intestinale Barriere [20] oder lymphatisches Gewebe [21] untersucht und charakterisiert.

Da das menschliche Mittelohr mit einem respiratorischem Epithel [22] sowie einem darüber liegenden Flimmerepithel, sekretorischen Zellen und Becherzellen [23]

ausgekleidet ist, könnte dieses eine ideale Interaktionsmöglichkeit mit Lektinen bieten. Aus diesem Grund ist das Ziel der vorliegenden Studie, durch den Einsatz verschiedener Lektine die Glykokalyx der MEM von Meerschweinchen zu charakterisieren, um dadurch einen für ein mögliches Lektin-vermitteltes Targeting geeigneten Liganden zu finden.

Zur Charakterisierung des Glykosylierungsmusters sollte die MEM mit ausgewählten Fluoreszein-markierten pflanzlichen Lektinen inkubiert und das Bindungsmuster quantitativ als auch visuell ausgewertet werden. Zuckerstrukturen wie N-acetyl-D-glucosamin und Sialinsäure [24] sollten durch Weizenagglutinin (WGA, wheat germ agglutinin) nachgewiesen werden. N-Acetyl-D-Glucosamin [25] Strukturen sollten auch durch die Bindung von Kartoffellektin (STA, solanum tuberosum lectin) bestätigt werden. α-L-Fucose [26] Ketten interagieren mit dem Ulex europaeus isoagglutinin I (UEA-I), während Schneeglöckchen-Lektin (GNA, Galanthus nivalis agglutinin) [27] spezifisch an Mannose bindet. Zusätzlich sollte Küchenlinsen-Lektin (LCA, lens culinaris agglutinin) eingesetzt werden, welches mit Galactosaminyl- bzw. α-Mannosyl-Resten interagiert [28]. Um die Spezifität der Wechselwirkungen näher charakterisieren zu können, wurde die Bindung kompetitiv mit den korrespondierenden Kohlenhydraten gehemmt.

Zusätzlich, um den Bindungsort zu lokalisieren, wurde das in der Histopathologie verwendete Alcian blue eingesetzt [29,30]. Durch diesen polyvalenten, basischen Farbstoff können oberflächlich vorhandene carboxylierte oder sulfatierte saure Mucopolysaccharide sowie Sialinmuzine (Glycoproteine) [31] sichtbar gemacht werden.

Durch die Bestimmung des Glycosylierungsmusters könnte eine neue Lektin-vermittelte Arzneiform entwickelt werden, die bei der Behandlung von OM durch eine längere Verweildauer in der Paukenhöhle zum wünschenswerten Therapieerfolg führt.

2. Ergebnisse und Diskussion

Es konnte im Rahmen der vorliegenden Diplomarbeit zur Publikation

“Determination of the glycosylation-pattern of the middle ear mucosa in guinea pigs”

von

E. Engleder, E. Demmerer, XY. Wang, C. Honeder, C. Zhu, C. Studenik, M. Wirth, C. Arnoldner, F. Gabor

durch Ausführung und Auswertung der beschriebenen Versuche maßgeblich beigetragen werden (siehe Anhang A).

Als Voraussetzung für ein Lektin-vermitteltes drug-targeting bei der häufig im Kindesalter auftretenden Otitis media (OM) wurde in dieser Arbeit das Glykosylierungsmuster der Mittelohrschleimhaut (MEM; middle ear mucosa) von Meerschweinchen näher charakterisiert. Zu Beginn der experimentellen Arbeit wurde die Viabilität der MEM nach unterschiedlichen Entnahmetechniken und nach zeitabhängiger Lagerung in verschiedenen Puffern bei zwei Temperaturstufen (4°C und 37°C) durch Zellkernfärbung mit Propidium-iodid kontrolliert.

Nach Optimierung der Färbetechnik, als auch Sicherstellung der Viabilität der MEM durch die eingesetzten Puffer, wurde das Glykosylierungsmuster an Hand der Interaktion mit fünf Fluoreszein-markierten, pflanzlichen Lektinen unterschiedlicher Kohlenhydratspezifität untersucht. Dazu wurde eine Lektin-Konzentration von 500 pmol/ml gewählt und die Bindungsrate mikroskopisch als auch quantitativ ermittelt. Für die quantitative Auswertung und den Vergleich der Zellinteraktionen untereinander wurden die Werte der gebundenen mittleren Fluoreszenzintensität (MFI) jedes Lektins auf einen Substitutionsgrad von einem Mol Fluoreszein pro Mol Lektin (F/P-Ratio) bezogen. Zusätzlich, um durch mögliche Überlagerung von Membranteilen bedingte hohe RFI-Werte zu erkennen, sowie die unterschiedliche Größe der Präparate zu berücksichtigen, wurden die RFI-Werte von Fluoreszein auf die mit Hoechst 33342 gefärbten Zellkerne bezogen und in %MFI (% of the mean MEM-associated fluorescence intensity) ausgedrückt.

Die Lektin-Bindungsstudien ergaben sowohl bei 4°C- als auch bei 37°C-Inkubation die höchste Bindungskapazität für WGA mit MFI-Werten von 2,32 ± 0,6% bzw. 2,55 ± 0,9%. Obwohl bei beiden Temperaturstufen WGA-Bindungskapazität am höchsten war, zeigten die übrigen eingesetzten Lektine im Mittel unterschiedliche Bindungsraten. Bei 4°C entsprach die MEM-assoziierte Lektinmenge der Reihenfolge WGA >> LCA > STA > UEA-I >> GNA. Im Gegensatz dazu bewirkte die Inkubation bei 37°C folgendes Ranking: WGA > UEA-I > STA > LCA > GNA. Bezogen auf die Lektin-Bindung wurden bei 4°C signifikante Unterschiede ($p < 0,05$) zwischen WGA und den anderen Lektinen sowie zwischen GNA und UEA-I, STA und LCA beobachtet. Im Gegensatz dazu war bei 37°C lediglich die Bindung von WGA und GNA signifikant unterschiedlich.

Die mikroskopische Auswertung ergab sowohl bei 4°C als auch bei 37°C ein wiederkehrendes Bindungsmuster von WGA, STA und in geringem Ausmaß auch LCA und UEA-I. Um auch mikroskopisch die Membran-Interaktion beurteilen und vergleichen zu können, wurden die Belichtungszeiten der F/P-Ratio angepasst. Auf Grund der höchsten Bindungskapazität von WGA bei beiden Temperaturstufen kann auf einen großen Anteil an frei zugänglichen Sialinsäureresten als auch auf das Vorhandensein von N-acetyl-D-glucosamin-Teilstrukturen an der Oberfläche der MEM geschlossen werden. Infolge der signifikant geringeren Bindungsrate von STA, einem N-Acetyl-D-glucosamin-spezifischen Lektin, der jedoch hohen Bindungsrate von WGA dürfte an der MEM ein wesentlich höherer Sialinsäureanteil vorliegen. Zusätzlich weist die ausgeprägte Bindungsrate von UEA-I als auch LCA auf zugängliche das vorhandene α-L-Fucose-Teilstrukturen sowie Galactosaminyl- und/oder Mannosereste in der MEM hin. Da die MFI der GNA-Bindung kaum über der Autofluoreszenz der MEM lag, ist die signifikant höhere ($p < 0,05$) Bindung von LCA bei 4°C auf eine Interaktion mit Galactosaminyl-Strukturen und nicht auf eine Bindung an α-Mannose-Reste zurückzuführen.

Um die Lektin-hältige Arzneiform stabil in der MEM zu verankern, ist die Spezifität der Interaktion des biorekognitiven Liganden am Wirkstoffträger mit der Membran ein wichtiger Parameter. Diese Spezifität wurde nur für WGA bestimmt, da dieses Lektin die höchste Bindungsrate erzielte. Durch Zugabe ansteigender Mengen des komplementären Zuckers Chitotriose konnte die Lektin-Bindung inhibiert und eine Abnahme der MEM-assoziierten Fluoreszenz beobachtet werden. Die Zugabe

steigender Konzentrationen (0-250 nmol/ml) des inhibierenden Trisaccharides bewirkte eine signifikante Inhibition der WGA-Bindung von bis zu 90%, was die hohe Spezifität der WGA-MEM beweist. Zusätzlich sollten durch Bindungsstudien mit Fluorescein-markiertem α-Lactalbumin unspezifische Protein-Membran Interaktionen erkannt werden. Da weder fluorimetrisch noch mikroskopisch eine Bindung des Proteins an die MEM detektiert werden konnte, unterstreicht dieser Nachweis zusätzlich die Spezifität der WGA-MEM-Bindung.

Zur Lokalisierung der Lektin-Bindung wurden mikroskopisch DIC- (differential interference contrast) und Fluoreszenzbilder der MEM-Oberfläche nach Lektinbindung aufgenommen. Der Vergleich zeigt eine bevorzugte Interaktion der Lektine mit den oberflächlich liegenden Zilienzellen. Um dies zu bestätigen wurden die sauren Mucopolysaccharide und Glycoproteine sowohl mit Alcian blue als auch mit dem Lektin F-WGA gleichzeitig gefärbt. Die Auswertung dieser Aufnahmen ergab für beide Färbemethoden auf derselben Ebene dieselben wiederkehrenden Muster. Um den Bindungsort weiter einzuschränken wurden Membranquerschnitte nach Färbung mikroskopisch analysiert. Diese Experimente ergaben, dass WGA mit der Oberfläche der Schleimhaut interagiert, da sich die Ebene der Zellkerne bis zu 6 µm unterhalb der Lektinbindungsorte befand.

Um auch eine mögliche Aufnahme von WGA in die Zelle und damit den potentiellen Transport von assoziierten Wirkstoffen in das Cytoplasma zu untersuchen wurden Internalisationsstudien mit F-WGA durchgeführt. Nach Bindung des Lektins an die Zellmembran bei 4°C (Pulse-Inkubation) und Entfernung des Lektinüberschusses wurde 60 Minuten bei 37°C (Chase-Inkubation) nachinkubiert. Dabei nahm die zellassoziierte MFI von 2,32 ± 0,6% auf 1,71 ± 0,6% ab, was einer Aufnahme von etwa 25% des ursprünglich Membran-gebundenen Lektins entsprechen könnte. Jedoch war keine Signifikanz zu den Messwerten der zellassoziierten MFI bei 37°C oder 4°C gegeben, sodass das Lektin nicht oder in vernachlässigbarer Menge in die MEM aufgenommen wird.

Zusammenfassend konnte in dieser Arbeit ein breites Spektrum an Methoden zur Charakterisierung der Bioadhäsion von biorekognitiven Liganden an die MEM von Meerschweinchen entwickelt werden. Die untersuchten Lektine unterschiedlicher Zuckerspezifität zeigten ein differenziertes Bindungsverhalten, wobei WGA sowohl bei 37°C als auch bei 4°C die höchste und auch eine hochspezifische Interaktion mit

der MEM aufwies. Durch Einsatz geeigneter Doppelfärbemethoden und Analyse von apikalen Membranabschnitten und Querschnitten konnte die Zelloberfläche als Bindungsort von WGA lokalisiert werden. Entsprechend der Ergebnisse von Puls-Chase Inkubationen wird nur ein vernachlässigbarer Anteil des Membran-gebundenen Lektins in die MEM aufgenommen.

Insgesamt konnte gezeigt werden, dass WGA als biorekognitiver und spezifisch bioadhäsiver Ligand sowohl für Polymerkonjugate als auch für partikuläre Arzneistoffsysteme zur Anreicherung von Wirkstoffen an der MEM bestens geeignet ist. Nach dieser ersten grundlegenden Studie bleibt es natürlich weiteren Arbeiten vorbehalten weitere wichtige Fragestellungen wie eine mögliche Änderung im Glykosylierungsmuster während einer OM, die Auswirkung des von Bakterien gebildeten Biofilms auf die Interaktion, oder auch den Einfluss der Mucus-Viskosität oder des Zilienschlages auf die Lektin-MEM Wechselwirkung zu untersuchen.

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Anhang A: PUBLIKATION

"Determination of the glycosylation-pattern of the middle ear mucosa in guinea pigs"

von

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to be submitted to the International Journal of Pharmaceutics.

Abstract:

Local intratympanic administration of antibiotics is aimed to improve therapy of Otitis media (OM), one of the most common diseases in pediatrics. Not only local administration, but additionally a prolonged contact time of the drug with the middle ear mucosa (MEM) is supposed to increase the therapeutic outcome. To approach this aim, plant lectins with different carbohydrate specificities are used to identify their utility as bioadhesive ligands as well as to investigate the glycosylation pattern of the MEM in guinea pigs. For that purpose, the viable MEM specimens were incubated at 4°C and 37°C with five different fluorescein-labeled plant-lectins and the lectin binding capacities were calculated from the MEM-associated relative fluorescence intensities (RFI). At both temperature levels F-WGA exhibited the highest binding rate whereas all other lectins interacted to a quite lower but different extent with the MEM. At 37°C the carbohydrate accessibility followed the order: sialic acid and N-acetyl-D-glucosamine > fucose > N-acetyl-D-glucosamine > α-mannose and galactosamine > mannose compared to the ranking at 4°C: sialic acid and N-acetyl-D-glucosamine >> α-mannose and galactosamine > N-acetyl-D-glucosamine > fucose >> mannose. According to competitive inhibition of binding with the corresponding carbohydrate F-WGA binding was inhibited up to 90% which confirms specificity of the F-WGA- MEM interaction. Internalization studies revealed that membrane bound F-WGA is not taken up into the cytoplasm. In order to localize and visualize the lectin-binding pattern the acidic mucopolysaccharides of the MEM were stained with alcian blue. The co-staining with F-WGA confirmed the lectin-binding on the MEM-surface by an overlay of the visible and the fluorescence pattern. Through the specific interaction of F-WGA with the MEM-surface this might be a possible drug carrier to prolong the residence time in the tympanic cavity and improve the treatment of OM. All in all, the specific interaction of F-WGA with the MEM-surface might be a promising strategy for successful treatment of OM.

1. Introduction

Otitis media (OM) is one of the most common inflammatory diseases of children and therefore the third most common reason for antibiotic therapy in pediatrics [Holstiege et at., 2013]. Although specific antibiotics are administered, the overall clinical effectiveness is limited due to low penetration of the drug to the middle ear mucosa (MEM) [Coates et al., 2008], inaccessibility of the bacteria within the grown biofilm [Hall-Stoodley et al., 2006] as well as low symptomatic amendment within the first 24 hours [Glasziou et al., 2004; Rovers et al., 2006]. To increase the therapeutic outcome and to prolong the contact time of the drug with the infected tissue drug loaded formulations such as thermosensitive hydrogels [Lee et al., 2004; Li et al., 2014; Honeder et al., 2014], ototopical drops [Kutz et al., 2013], implants [Goycoolea et al. 1992, 1994; Nether et al., 2004], micropumps [Lehner et al., 1997], intranasal drug delivery systems [Chandrasekhar et al., 2004], coated middle ear prostheses [Lensing et al., 2013; Ehlert et al., 2013; Hesse et al., 2012], and pellets [Daniel et al., 2012] were developed. Although there are many different therapeutic approaches, a local intratympanic therapy seems to be most beneficial for the treatment of OM as a decrease in side effects provoked by systemic therapy as well as an increase of the compliance of the young patients will be expected. Nevertheless, the local intratympanic therapy is limited by unfavorable anatomical conditions. The nasopharynx is connected with the tympanic cavity that can lead to rapid drainage of intratympanally administered solutions and suspensions. To avoid Eustachian drainage and to concurrently prolong the contact time of the drug we propose bio-adhesive carrier systems interacting with the MEM. As the middle ear is lined with a modified respiratory epithelial layer [Hentzer et al., 1972] and comprises, among others, ciliated, secretory as well as goblet cells [Lim et al., 1972] the carbohydrates of the glycocalyx at the membrane of these cells can be exploited as bioadhesive sites for targeted delivery. Carbohydrate-binding proteins such as plant lectins interacting with certain sugar residues on the cell surface can function as a ligand. This bioadhesion concept of lectin-mediated targeting has been already reported for overcoming several biological barriers [Bies et al., 2004; Wirth et al., 2002], such as the intestinal epithelium [Gabor et al., 1998], the urothelium [Plattner et al., 2008; Neutsch et al., 2013], the blood-brain barrier [Plattner et al., 2009], and the lymphoid tissue [Diesner et al., 2012].

As a first step towards putting this concept into practice the glycosylation pattern of the MEM has to be elucidated, which is not reported until now to the best our knowledge. To identify accessible carbohydrate moieties and vice versa appropriate bioadhesive ligands, the interaction of MEM isolated from guinea pigs with a panel of fluorescent labeled lectins with different carbohydrate specificities was investigated: the wheat germ agglutinin (WGA) from *Triticum vulgare* binding to N-acetyl-D-glucosamine and sialic acid [Goldstein et al., 1986], the lectin from furze seeds (*Ulex europaeus* isoagglutinin I, UEA-I) which interacts with α-L-fucose-containing carbohydrates [Gürtler et al., 1978], the mannose-specific *Galanthus nivalis* agglutinin (GNA) [Van Damme et al., 1987], the *Solanum tuberosum* lectin (STA) from potato tubers binding to N-acetyl-D-glucosamine [Allen et al., 1973], and the lentil lectin from *Lens culinaris* (LCA) recognizing galactosaminyl-/α-mannosyl-residues [Flika et al., 1978]. Ongoing from cytoadhesion experiments at 4°C and cytoinvasion assays at 37°C, the specificity of interaction will be described. Additionally, co-localization of lectin-interacting carbohydrates and acidic mucopolysaccharides was applied to identify the lectin binding sites at the MEM.

All in all, this study is aimed to roughly characterize the carbohydrate pattern of the MEM and to identify ligands for glycotargeting as a basis for the development of bioadhesive antibiotic formulations to improve therapy of OM.

2. Materials and methods

2.1. Materials

The fluorescein-labeled lectins from *Triticum vulgare* (WGA; wheat germ agglutinin, molar ratio fluorescein/protein (F/P) = 4.5), *Solanum tuberosum* (STA; F/P = 3.0), *Ulex europaeus* (UEA-I, isoagglutinin I; F/P = 2.9), *Galanthus nivalis* (GNA; F/P = 5.5), and *Lens culinaris* (LCA; F/P = 3.4) were purchased from Vector Laboratories (Burlingame, CA, USA). Hoechst 33342 trihydrochloride trihydrate was obtained from Invitrogen (Vienna, Austria). Alcian blue and Chitotriose were from Sigma-Aldrich (Vienna, Austria). Fluorescein-labeled α-lactalbumin was acquired from Molecular Probes (Eugene, Oregon, USA). All other chemicals were bought from Sigma Aldrich and of analytical grade.

2.2. Lectin-binding capacity of the MEM

Immediately after sacrificing the guinea pig the bullas were dissected and opened carefully. After rinsing the MEM with 0.9% NaCl the bullas were fixed with the auditory canal upside and the mucosa was incubated with 500 µl solution of fluorescein-labeled lectins (500 pmol/ml 0.9% NaCl) for 30 min at 4°C or 37°C. Unbound lectin was removed by washing the cell layer 5 times with 800 µl saline and the nuclei were stained by incubation with 500 µl solution of Hoechst 33342 (0.1 mg/ml 0.9% NaCl) for 10 min at 37°C. The specimen was washed again thoroughly and the staining pattern of the MEM was fixed by incubation in ice cold MeOH at -20°C for 20 min. After rehydration in 0.9% NaCl at room temperature for another 20 min, the MEM was harvested and mounted on a slide in FluorSave™ for visualization and quantification.

As a control to estimate nonspecific binding, samples prepared as described above were treated with a solution of F-lactalbumin instead of the lectins.

2.3. Specificity of lectin-binding

To investigate the specificity of the lectin-cell interaction competitive inhibition experiments using the complementary carbohydrates were performed. After washing the bulla with 0.9% NaCl the MEM was incubated with a freshly prepared mixture of 250 µl solution of the complementary carbohydrate (0-500 nmol/ml) and 250 µl solution of the corresponding lectin (1000 pmol/ml) for 30 min at 4°C. After removal

of non-bound lectin and soluble carbohydrate-lectin complexes by thorough washings and preparation of the MEM the cell-bound fluorescence was determined as described below.

2.4. Lectin-uptake by the MEM

In order to find out, whether MEM-bound lectin is taken up into the cells, a pulse-chase protocol was performed: the MEM was incubated 500 µl solution of fluorescein-labeled lectins (500 pmol/ml 0.9% NaCl) for 30 min at 4°C followed by removal of unbound lectin by five washings with saline. The cell-bound lectins were allowed to be internalized during the chase incubation at 37°C for another 60 min. Subsequently, the MFI was determined as described below.

2.5. Staining of acidic components of the mucosa

After fixing the ear with the auditory canal in an upright position, the bulla was filled with 500 µl 3% acetic acid and incubated for 3 minutes. This solution was replaced by 500 µl alcian blue solution (10 mg/ml in 3% acetic acid) and removed after 30 min incubation at room temperature [Sheehan et al., 1980; Bancroft et al., 1982]. The MEM was washed 5 times with aqueous 0.9% NaCl solution and then the lectin binding capacity was determined at 37°C as described above.

2.6. Semi-quantitative measurement of fluorescence

The relative cell-layer-associated fluorescence intensity (RFI) of the fluorescein-labeled lectins and the Hoechst 33342 stained nuclei were determined using a fluorescence microplate reader (TECAN, Infinite M200, Austria) at an excitation/emission of 485/525 nm and 365/450 nm, respectively. The slides were mounted at a defined position and the laser was adjusted to read one 3x3 mm square after another throughout the whole area of the fixed slide. Cell-layers incubated with buffer served as a control for autofluorescence of cells and slides.

To guarantee comparability of the results and to consider the influence of uneven and folded surfaces on quantum yield, the RFI of the nuclei stained with Hoechst 33342 was chosen as a measure for the amount of tissue. The fluorescein-readouts were related to this area and expressed as a percentage.

2.7. Microscopy

To visualize adhesion of the different lectins to the MEM, fluorescence images of the prepared slides were acquired with a Zeiss Axio Observer.Z1 microscopy system equipped with LED illumination system “Colybri” (Zeiss, Göttingen, Germany). For comparability of the images, the excitation exposure time of each lectin was related to an apparent conjugation number of 1 mol fluorescein per mol of lectin by considering the F/P-ratio.

Histological images were obtained using a Nikon Eclipse 50i microscope equipped with an EXFO X-Cite 120 fluorescence illumination system. The images of the FITC-labeled lectins at an excitation/emission of 465-495/515-555 nm as well as the transmitted light images were acquired at 20x and 40x magnification and processed using Lucia G v5.0 software for evaluation.

2.8. Statistics

The integrated analysis tools of Microsoft Excel® were used to carry out statistical analyses. The hypothesis test was made by comparing two means from independent samples, among two data sets (t-test). Values of $p < 0.05$ were considered as significant. All experiments were performed at least three times.

3. Results

To guarantee that the upcoming experiments are performed with viable MEM cells different preparation techniques were tested in preliminary assays using propidium iodide staining of nuclei as a selection criterion. In general, incubation in presence of PBS resulted in immediate loss of viability. Additionally, detachment of the MEM from the bone matrix followed by incubation with ligands yielded non-utilizable specimens due to folding of the tissue and uneven staining. Only incubation of the bone attached mucosa in presence of saline and subsequent isolation of the MEM just before analysis yielded reliable results.

3.1. Lectin-binding capacity

Preliminary studies were aimed to select the appropriate concentration range for the lectin binding studies. Whereas F-WGA seemed to oversaturate the specimens at concentrations higher than 1000 pmol/ml, 500 pmol/ml were still sufficient to saturate potential binding sites. Thus this concentration was applied to investigate the glycosylation pattern of the MEM from guinea pigs by use of five fluorescein-labelled lectins with different carbohydrate specificities.

To allow compatibility of the data, different issues were considered for calculation: (i) As the degree of fluorescein-substitution differs between the lectins, the mean cell-bound fluorescence intensity (FI) of each lectin was related to an apparent conjugation number of 1 mol fluorescein per mol lectin according to the fluorescein/protein ratio. (ii) As the size of the specimens is different and sometimes parts of the collected MEM were overlapping, the highest FI of squares with stained nuclei was set 100% and only squares were considered with a FI higher than the autofluorescence of the cells. (iii) Only the FI of cell-associated lectins of nuclei positive squares was used, related to the FI of the stained nuclei in these squares and expressed as a percentage.

Upon incubation at 4°C WGA exhibited the highest binding rate with 2.32 ± 0.6% mean MEM-associated FI (MFI) and F-GNL the lowest one close to the autofluorescence of the MEM (Fig. 1A). The other three lectins was similar being about 0.5% MFI, so that the MEM-associated fluorescence intensities followed the order: WGA >> LCA > STA > UEA-I >> GNA. Besides, the interaction of WGA and all other lectins as well as that between GNL and UEA-I, STA as well as LCA the was significantly different ($p < 0.05$).At 37°C the highest MEM-association capacity was

again observed in case of WGA amounting to 2.55 ± 0.7% MFI (Fig. 1B). Nevertheless, this interaction was only significantly different to that of GNA. At the mean, some places changed in the ranking in comparison to 4°C resulting in the following order: WGA > UEA-I > STA > LCA > GNA.

3.2. Microscopical visualization of the binding pattern

The microscopic visualization of the lectin-MEM interaction confirmed the results of the quantitative assay at both temperature levels as the FI of the images decreased according to the ranking described above (Fig.2).Interestingly, differences in the staining pattern are observed: the N-acetyl-D-glucosamine interacting lectins WGA and STA yielded colored clusters and WGA showed additional less intense and diffuse staining. LCA was rather evenly distributed throughout the MEM-surface and UEA-I-binding was lowest but still visible. In contrast, the binding of GNA could not be observed microscopically.

As WGA proved to highly interact with the MEM, this interaction was elucidated in more detail. The overlay of differential interference contrast (DIC) images and fluorescence images revealed that the WGA-binding pattern coincides with the ciliated cell surface (Fig. 3). Although the exposure time was extended to 3 sec binding of F-LCA and F-GNA to ciliated areas was not detectable. To get some further evidence for WGA-binding to the cilia, a z-stack of images was collected. At the level of the nuclei (Fig. 4 A) no fluorescence of bound WGA was observed. Moving the focus plane 6µm higher towards the apical face (Fig. 4 B), the nuclei were still visible and a rather weak scattered diffuse fluorescence of WGA is detectable. Analyzing the image acquired another 6µm higher revealed diffuse blue fluorescence but a sharp F-WGA staining of finger-like structures at the MEM (Fig. 4 C), which disappeared again by further rising the focus plane (Fig. 4 D). This confirms that WGA predominantly interacts with protruding elements of the MEM such as the cilia. Additionally, as displayed in Fig.5, co-localization of acidic glycans and the binding site of WGA by co-staining with Alcian blue and F-WGA revealed a punctuate pattern on the MEM indicative for acidic polysaccharides as a lectin combing site on the cilia of the MEM.

3.3. Specificity of lectin-binding

Contribution of non-specific protein-protein interactions to the lectin-MEM interaction was investigated by binding studies with F-lactalbumin(Permyakov, 2005). Even in presence of high amounts of lactalbumin up to to 500 pmol/ml, no interaction could be detected.

As only WGA considerably interacted with the mucosa, the specificity of the interaction was elucidated by competitive inhibition of lectin binding sites at the mucosa by addition of the best fitting complementary carbohydrate N, N',N''-triacetyl-chitotriose. The MFI of MEM-bound WGA decreased with increasing amounts of the corresponding carbohydrate up to 90% (Fig. 6). The values were significantly ($p<0.05$) and confirm high specificity of the WGA-MEM interaction since the adhesion was inhibited by a defined molecule.

3.4. Uptake of WGA into MEM-cells

According to the observation that the mean MFI of MEM-bound WGA was slightly, but not significantly, higher at 37°C than at 4° the lectin might be bound and taken up into the cell. For that purpose, first, the lectin was bound to the cell-membrane at 4°C. At this temperature level the fluidity of the cell membrane and the metabolism is reduced and energy consuming mechanisms like active transport are repressed. After removal of non-bound lectin, the cells were incubated in a second step at 37°C to allow for energy dependent uptake processes. Due to shielding of the fluorescence of internalized ligand, the MFI is expected to decrease. Although the MFI was 2.32 ± 0.6% after incubation at 4°C and decreased to 1.71 ± 0.6% after incubation at 37°C for 60 min, again the differences were not significant (data not shown). Thus, internalization of membrane-associated lectin could not be confirmed.

4. Discussion

In an effort to identify bioadhesive or even cytoinvasive ligands for improved drug delivery in the middle ear, the glycosylation pattern of the MEM of guinea pigs, an approved model for research of OM [Zak et al., 1999], was systematic characterized via detailed analysis of the binding capacities of selected fluorescein-labeled lectins with different carbohydrate binding specificities.

For quantitation of the lectin-binding rate and comparability of results, the fluorescein density of the lectins as well as the fluorescein intensity of stained nuclei as a measure for the tissue were considered for calculation. According to the results, the glycocalyx of the MEM contains highest numbers of accessible sialic acid and N-acetyl-D-glucosamine moieties and minor amounts of fucosyl- or mannosyl-residues. In case of 37°C, however, the glycosylation pattern changed following the order: sialic acid and N-acetyl-D-glucosamine > fucose > N-acetyl-D-glucosamine > α-mannose and galactosamine > mannose. The different glycosylation pattern at the two temperature levels might be due to varied viscosity of the mucus [Kočevar-Nared et al., 1997] which seems to influence the accessibility of the carbohydrate residues. At body temperature 37°C the MEM contains about 3.17-fold higher amounts of fucose and mannose as well as 1.73-fold more N-acetyl-D-glucosamine residues. The number of sialic acid and N-acetyl-D-glucosamine or α-mannose and galactosamine moieties were comparable with those at 4°C increasing 1.10- and 1.15-fold, respectively.Since both, WGA and STA interact with N-acetyl-D-glucosamine, but WGA additionally with sialic acid, the 3.27 fold higher binding rate of WGA is indicative for presence of high amounts of sialyl-residues. This observation was additionally confirmed microscopically by co-localisation of WGA binding sites and acidic mucopolysaccharides.

For drug delivery to the middle ear not only the binding rate but also the specificity of the interaction is another important parameter. According to competitive inhibition studies WGA specifically interacted with the MEM as indicated by up to 90% inhibition. Additionally, non-specific protein-protein interactions negligibly contribute to lectin-MEM binding. Out of these reasons WGA is an interesting ligand for a lectin-mediated drug delivery to the middle ear. Although the MFI of WGA decreases by 25% only at the mean by increasing the temperature from 4°C to 37°C, cytoinvasion of the bioadhesive ligand could not be confirmed. It should be considered, however, that in case of OM the drug should preferably interact after local administration in the

tympanic cavity with the cause of disease, the bacteria. Nevertheless, uptake into the MEM-cells might be beneficial to combat also bacteria hidden in the cytoplasm.

The quantitation of lectin binding-efficiency is confirmed qualitatively by fluorescence microscopy revealing the sialyl- and N-acetyl-D-glucosamine specific WGA as the most efficient ligand. According to the literature, the composition of the glycocalyx is altered during OM due to upregulation of mucin genes [Ohashi et al., 1989]. Since mucin genes encode among others for sialic acid [Lin et al., 2001], the number of potential WGA-binding sites might increase even in case of inflammation and improve the residence time in the tympanic cavity and the efficiency of WGA-grafted formulations. For this reason, an

Applying double staining techniques, the cilia of the MEM containing no nucleus [Hentzer et al., 1976], are identified as a binding site for WGA. This aspect might be beneficial for lectin-mediated drug delivery since mucociliary clearance might decrease the efficacy of non-adhesive formulations. Although the ex-vivo studies were performed at body temperature and with MEM being stuck on the bony capsule of the middle ear, the motion of the cilia might be different in-vivo. However, according to the literature the ciliary motion is diminished [Ogasawara et al., 2007] during inflammation and might not influence the lectin-MEM interaction.

Another aspect to be considered for use of WGA-functionalized carrier systems for OM-therapy is the formation of a biofilm consisting of bacteria, which overlays the MEM [Post et al., 2004]. Consequently, the biofilm can limit accessibility of the MEM and block the docking sites for WGA so that the carrier cannot interact. However, former studies demonstrate that the biofilm is stained with WGA [Thornton et al., 2013]. Thus, even the biofilm might be an additional potential bioadhesive site for WGA-grafted drug delivery systems for therapy of OM.

5. Conclusion

All in all, the screening of the glycosylation pattern of the MEM by use of lectins with different carbohydrate specificity revealed that sialic acid and N-acetyl-D-glucosamine are the most abundant and accessible binding sites of the MEM. Consequently, among the lectins under investigation, WGA emerged as the most promising ligand for drug carrier systems interacting with the cilia of the MEM. Thus, the concept of lectin-mediated bioadhesive drug delivery is proposed as a platform for local intratympanic therapy, which offers prolonged residence time, shortened diffusional pathways, and increased concentration gradient that altogether result in improved efficacy of drugs.

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Figures and Tables

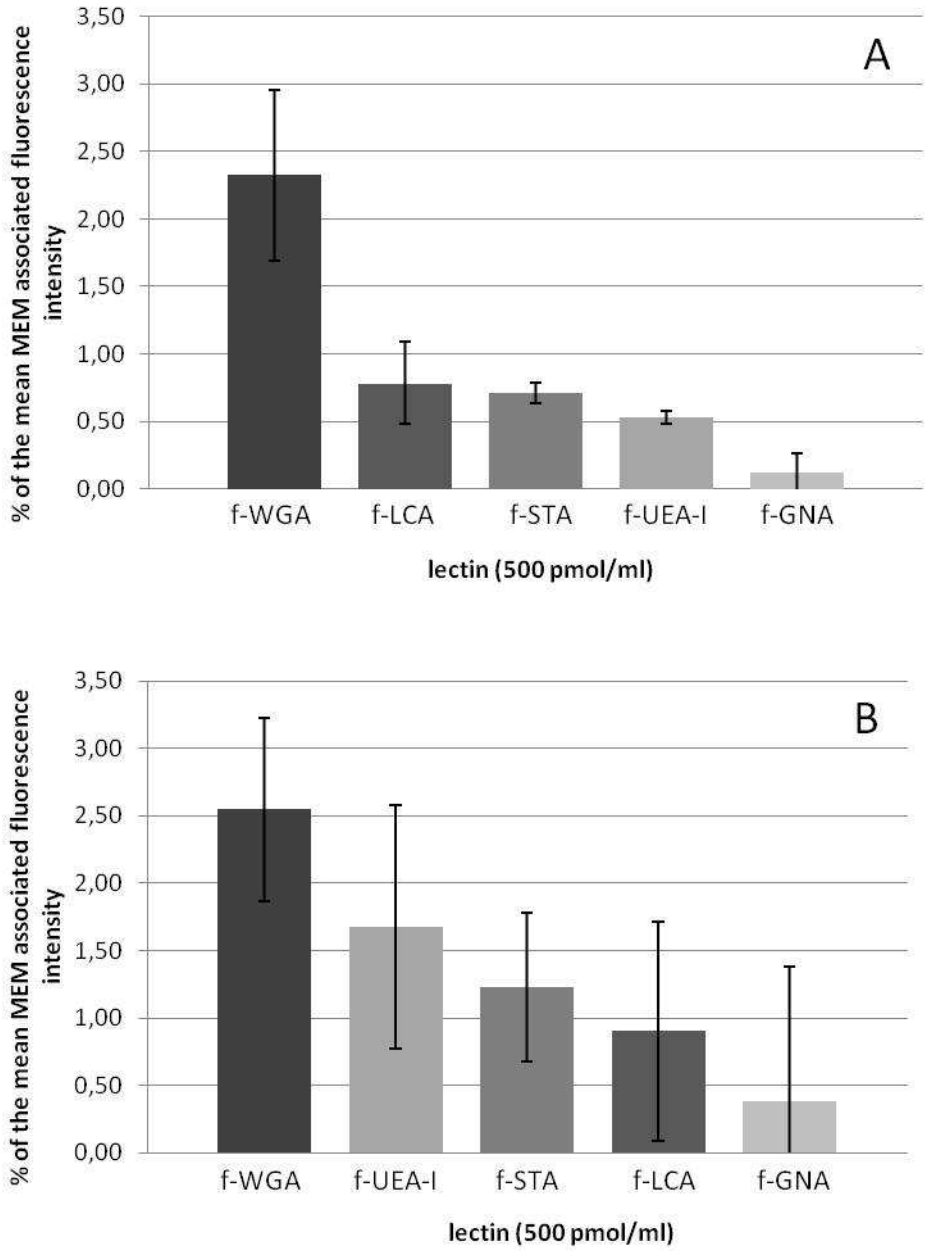

Fig.1.: Association of lectins with the MEM at 4°C (A) or 37°C (B). For comparability, the cell-associated fluorescein intensity was related to an apparent F/P-ratio of 1 as well as to the fluorescence intensity of stained nuclei (mean ± SD, n = 3).

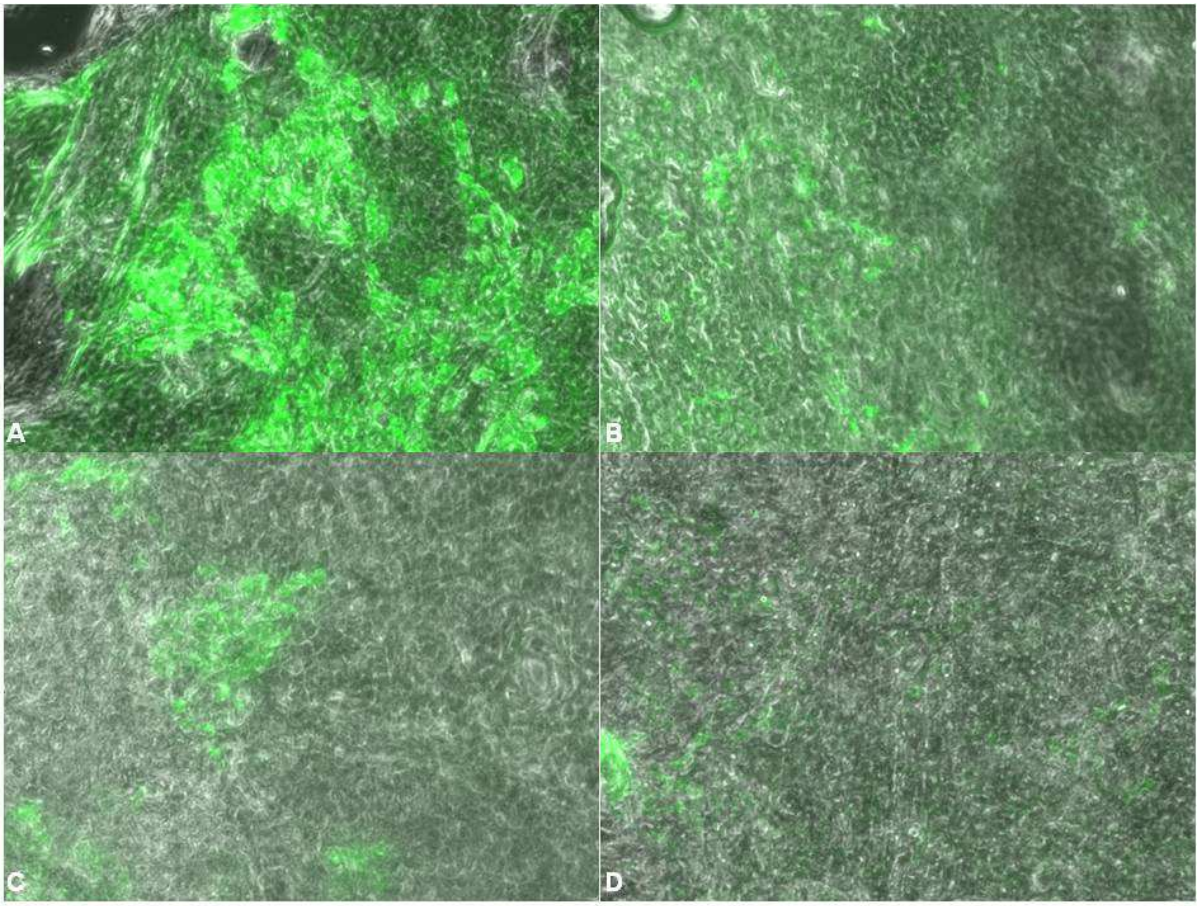

Fig.2.: Microscopical visualization and Comparison of the lectin binding pattern and intensity of F-WGA (A), F-LCA (B), F-STA (C) and F-UEA-I (D) after incubation at 4°C by overlaying fluorescence and DIC images. For comparability, the exposure time was correlated to the F/P-ratio of the lectins. Images were acquired at 20x magnification.

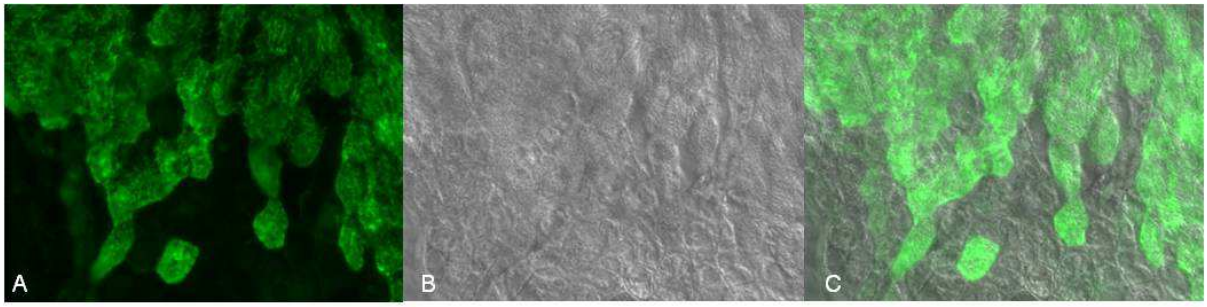

Fig.3.: Identification of cilia as a binding site for WGA by fluorescence imaging (A) DIC-imaging (B) and overlay of both (C). Images were acquired at 63x magnification.

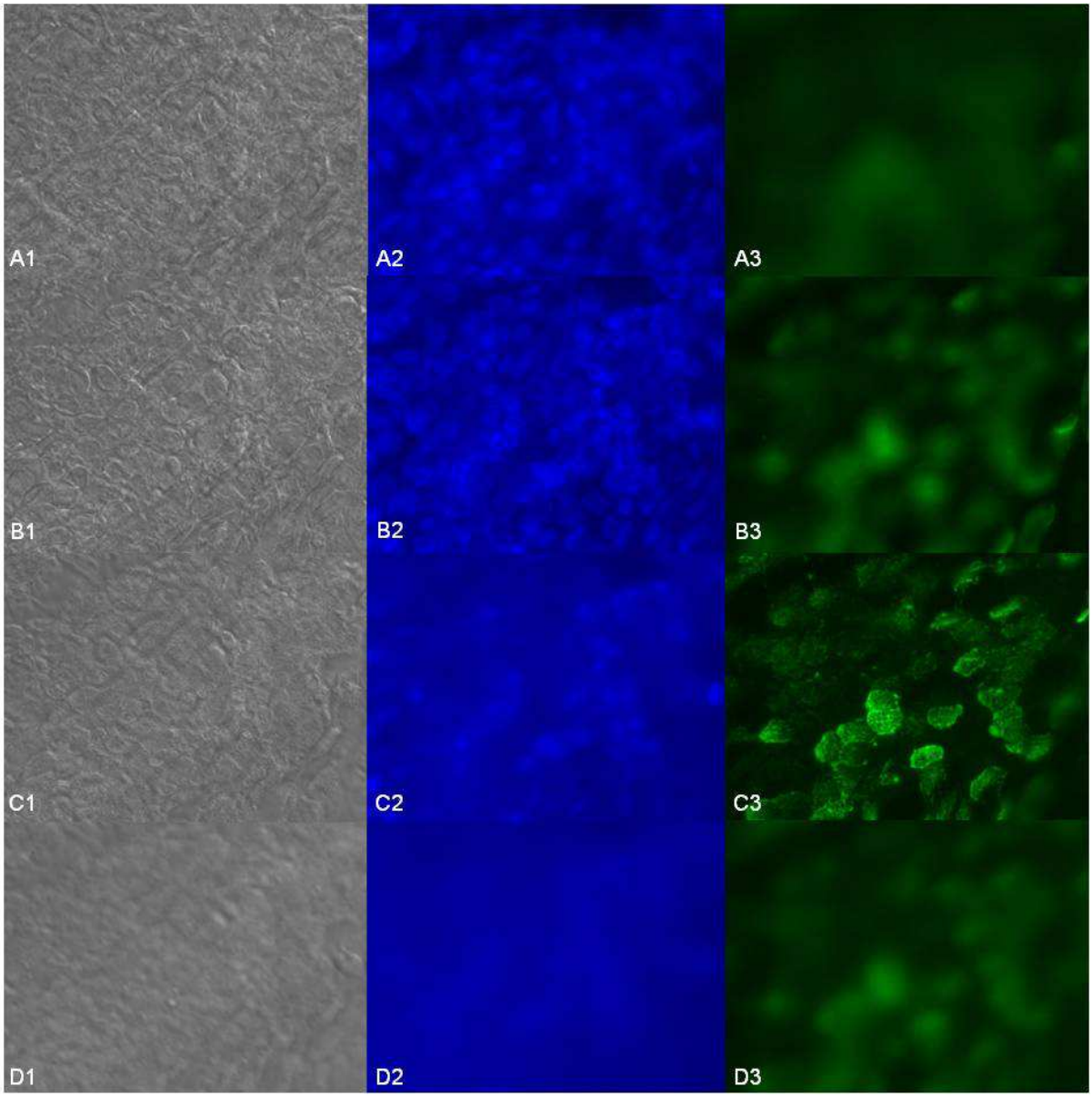

Fig.4.: Z-stack of DIC (1), nuclei (2, stained blue) and F-WGA (3, green) at different levels of the MEM: 0 µm (A), 6 µm (B), 12 µm (C) and 18 µm (D). Images were acquired with 63x magnification.

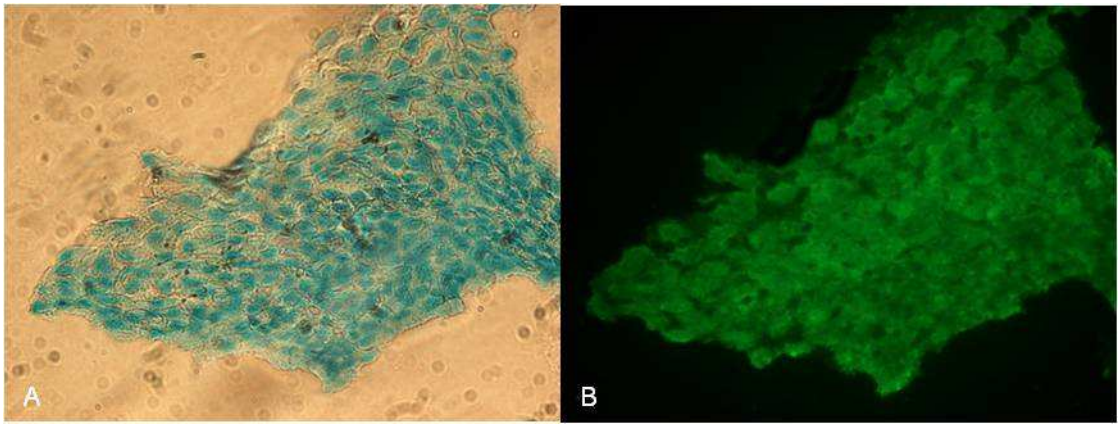

Fig.5.: Co-staining of the acidic mucopolysaccharides with Alcian blue (A) and sialyl- as well as N-acetyl-glucosaminyl residues with F-WGA (B) of the MEM. Images were acquired at 40x magnification.

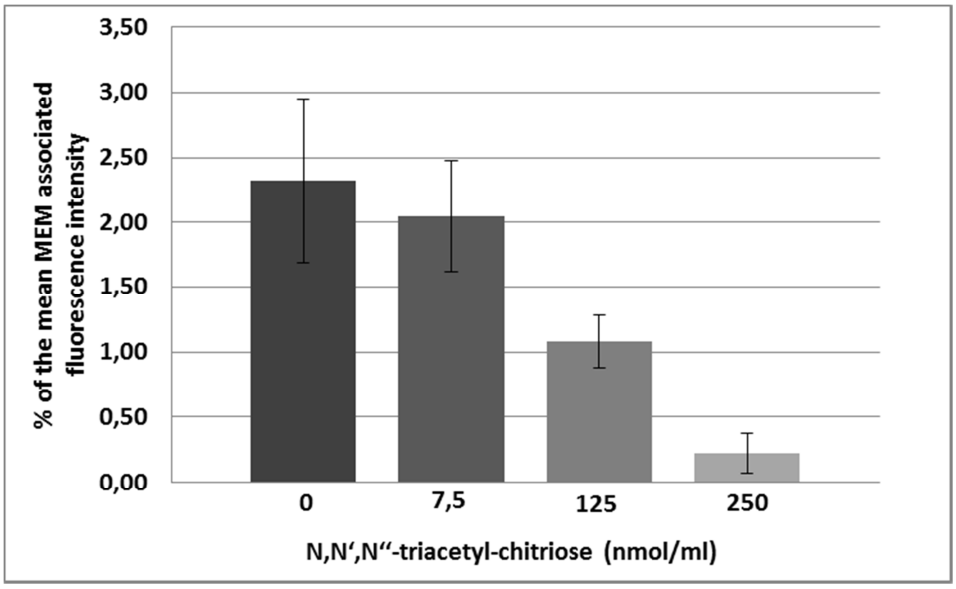

Fig.6.: Competitive inhibition of WGA-binding to the MEM by addition of increasing amounts of the complementary carbohydrate.

Zusammenfassung

Otitis media (OM) ist einer der häufigsten Gründe für eine Antibiotika-Therapie in der Pädiatrie, jedoch besitzt die derzeit angewandte Behandlung eine geringe klinische Effektivität. Eine lokale Therapie direkt im Mittelohr, die zu einer längeren Verweildauer des Wirkstoffes in der Paukenhöhle führt, könnte die OM-Therapie verbessern. Um hier einen optimalen Effekt zu erzielen und den Wirkstoff durch Bioadhäsion an der Mittelohrschleimhaut (MEM) zu immobilisieren, wurde in der vorliegenden Arbeit das Glycosylierungsmuster der MEM von Meerschweinchen an Hand von fünf Fluoreszein-markierten Pflanzenlektinen mit unterschiedlichen Zuckerspezifitäten untersucht. Zunächst wurden Färbe- sowie Entnahmetechniken optimiert und die Viabilität der MEM durch Propidium-iodid-Färbung gesichert. Die Lektinbindungsstudien ergaben sowohl bei 37°C als auch bei 4°C, das Weizenlektin (WGA) die höchste Bindungskapazität besitzt. Insgesamt war die Reihenfolge der zugänglichen Monosaccharide bei 4°C-Inkubation Sialinsäure and N-acetyl-ᴅ-glucosamine > α-mannose and Galactosamine > N-acetyl-ᴅ-glucosamine > Fucose > Mannose die sich durch Inkubation bei 37°C änderte und folgende Reihung zeigte: Sialinsäure und N-acetyl-ᴅ-glucosamine > Fucose > N-acetyl-ᴅ-glucosamine > α-mannose and Galactosamine > Mannose. Die Spezifität der WGA-MEM-Interaktion wurde durch kompetitive Inhibition mit dem komplementären Zucker N,N´,N´´-triacetyl-chitotriose untersucht und ergab eine Inhibition von mehr 90% und damit hohe Spezifität. An Hand eines „Pulse-Chase“-Protokolls wurde auch die mögliche Aufnahme des Membran-gebundenen Lektins untersucht, die jedoch keine nennenswerte Internalisation von WGA ergab. In Co-Lokalisationsstudien konnte nachgewiesen werden, dass das Lektin an saure Mucopolysaccharide der Glycocalyx bindet und bevorzugt mit den Zilien der MEM interagiert. Insgesamt ist die Kenntnis des Glykosylierungsmusters der MEM Voraussetzung für ein Zucker-Targeting und es konnte das Potential von WGA und dem Kartoffellektin (STA) als vielversprechende Liganden für ein Lektin-vermitteltes „Drug-Delivery“ zur Behandlung von OM gezeigt werden.

Lebenslauf

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