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

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ABSTRACT

In the present study the glycosylation pattern of the middle ear mucosa (MEM) of guinea pigs, an approved model for middle ear research, was characterized with the purpose to identify bioadhesive ligands which might prolong the contact time of drug delivery systems with the middle ear mucosa (MEM). To assess the utility of five fluorescein labeled plant lectins with different carbohydrate specificities as bioadhesive ligands, viable MEM specimens were incubated at 4 °C and the lectin binding capacities were calculated from the MEM-associated relative fluorescence intensities. Among all lectins under investigation, fluorescein-labeled wheat germ agglutinin (F-WGA) emerged as the highest bioadhesive lectin. In general, the accessibility of carbohydrate moieties of the MEM followed the order: sialic acid and *N*-acetyl-D-glucosamine (WGA) >> mannose and galactosamine (*Lens culinaris* agglutinin) > *N*-acetyl-D-glucosamine (*Solanum tuberosum* agglutinin) > fucose (*Ulex europaeus* isoagglutinin I) >> terminal mannose α-(1,3)-mannose (*Galanthus nivalis* agglutinin). Competitive inhibition studies with the corresponding carbohydrate revealed that F-WGA-binding was inhibited up to 90% confirming specificity of the F-WGA–MEM interaction. The cilia of the MEM were identified as F-WGA binding sites by fluorescence imaging as well as a z-stack of overlays of transmission, F-WGA- and nuclei-stained images of the MEM. Additionally, co-localisation experiments revealed that F-WGA bound to acidic mucopolysaccharides of the MEM. All in all, lectin-mediated bioadhesion to the MEM is proposed as a new concept for drug delivery to prolong the residence time of the drug in the tympanic cavity especially for successful therapy for difficult-to-treat diseases such as otitis media.

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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 al., 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 (Post et al., 2004) as well as low symptomatic amendment within the first 24 h (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; Goycoolea and Muchow, 1994; Nether et al., 2004), micropumps (Lehner et al., 1997), intranasal drug delivery systems (Chandrasekhar and Mautone, 2004), coated middle ear prostheses (Lensing et al., 2013; Ehlert et al., 2013; Hesse et al., 2013), 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 in compliance of the young patients will be expected. Nevertheless, the local intratympanic therapy is limited by unfavorable anatomical

Abbreviations: OM, otitis media; MEM, middle ear mucosa; WGA, wheat germ agglutinin; STA, *Solanum tuberosum* lectin; UEA-I, *Ulex europaeus* isoagglutinin I; LCA, *Lens culinaris* agglutinin; GNA, *Galanthus nivalis* agglutinin; MFI, mean fluorescence intensity.

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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 bioadhesive carrier systems interacting with the MEM. As the middle ear is lined with a modified respiratory epithelial layer (Hentzer, 1984) and comprises, among others, ciliated, secretory as well as goblet cells (Lim and Shimada, 1972), the carbohydrates of the glycocalyx at the membrane of these cells might be exploited as bioadhesive sites for glycotargeted 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., 2010), and the lymphoid tissue (Diesner et al., 2012).

As a first step toward putting this concept into practice the glycosylation pattern of the MEM has to be elucidated, which is not reported until now to the best of 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 and Poretz, 1986), the lectin from furze seeds (*Ulex europaeus* isoagglutinin I, UEA-I) which interacts with α-L-fucose-containing carbohydrates (Gürtler, 1978), the α-1,3-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 and Neuberger, 1973), and the lentil lectin from *Lens culinaris* (LCA) recognizing galactosaminyl-/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-localisation 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.

2. Materials and methods

2.1. Materials

The fluorescein-labeled lectins from *T. vulgare* (WGA; wheat germ agglutinin, molar ratio fluorescein/protein (F/P) = 4.5), *S. tuberosum* (STA; F/P = 3.0), *U. europaeus* (UEA-I, isoagglutinin I; F/P = 2.9), *G. 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 *N*,*N*′,*N*″-triacetyl-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 were 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 mounted 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 500 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 carefully removed from the bulla and mounted on a slide in FluorSave™ for visualization and quantification. To allow comparability of the data, different issues were considered for calculation: (i) since the degree of fluorescein-substitution differs between the lectins, the MFI 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 MFI of squares with stained nuclei was set 100% and only squares were considered with a MFI higher than the autofluorescence of the cells. (iii) Only the MFI of cell-associated lectins of nuclei positive squares was considered, related to the MFI of the stained nuclei in these squares, and expressed as a percentage.

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 F-WGA–cell interaction competitive inhibition experiments using the complementary carbohydrate *N*,*N*′,*N*″-triacetyl-chitotriose 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 F-WGA (500 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 the MEM-bound lectin is taken up into the cells, a pulse-chase protocol was performed: the MEM was incubated with 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 washing 5 times with saline. The cell-bound lectins were allowed to be internalized during the chase period at 37 °C for another 60 min. Subsequently, the mean MEM-associated fluorescence intensity (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 min. 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 and Hrapchak, 1980; Bancroft and Stevens, 1982). The MEM was washed 5 times with aqueous 0.9% NaCl solution and after staining with F-WGA as described above the lectin binding capacity was visualized by microscopy.

2.6. Semi-quantitative determination of fluorescence intensity

The relative cell-layer-associated fluorescence intensity of the fluorescein-labeled lectins and the Hoechst 33342 stained nuclei

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