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In situ characterization of glycans in the urothelium of donkey bladder: Evidence of secretion of sialomucins

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ABSTRACT

The glycoprotein pattern was investigated by lectin histochemistry in the urothelium lining the urinary bladder of the donkey *Equus asinus*. Tissue sections were stained with a panel of twelve lectins, in combination with saponification and sialidase digestion (K-s). The urinary bladder urothelium has three distinct layers from the basal zone to the lumen consisting of basal, intermediate and superficial cells (umbrella cells). Cytoplasm of basal cells reacted with SNA, PNA, K-s-PNA, GSA I-B_4 and Con A showing glycans ending with Neu5Acα2,6Gal/GalNAc, Neu5AcGalβ1,3GalNAc, αGal and with terminal/internal αMan. The cytoplasm of umbrella cells displayed an increase of Neu5AcGalβ1,3GalNAc and the appearance of Neu5AcGalβ1,3GalNAc, Neu5acα2,3Galβ1,4GlcNAc and Neu5AcGalNAc residues (MAL II, K-s-SBA and K-s-HPA staining). Scattered umbrella cells were characterized by glycans terminating with GalNAc binding DBA, SBA and HPA. The mucosa forms folds with a crypt-like appearance where the urothelium shows a different pattern of glycans. The bladder luminal surface stained with K-s-PNA, K-s-DBA, KOH-s-SBA, and K-s-HPA displaying a coating of sialoglycoproteins belonging to O-linked glycans (typical secretory moieties). These findings show that different glycosylation patterns exist along the donkey bladder urothelium, and different sub-populations of umbrella cells are present secreting the sialoglycans which constitute the protective gel layer lining the bladder.

Introduction

The urinary bladder is lined by the urothelium, a transitional epithelium which has three distinct layers consisting from the basal lamina to the lumen of basal cells, intermediate cells and superficial cells (so-called umbrella cells). In normal urothelium, differentiated superficial cells gradually develop from basal and intermediate cells (Veranic et al., 2004). The highly differentiated umbrella cells are characterized by a sub-apical trajectorial network of cytokeratins (Veranic and Jezernik, 2002) and membrane-protein plaques, which cover their apical surface (Hicks, 1965, 1966, 1975; Kachar et al., 1999; Koss, 1969; Staehelin et al., 1972; Kreft et al., 2010; Hudoklin et al., 2011).

The healthy mammalian urothelium serves as a permeability barrier and provides protection from environmental chemicals and microbes (Callahan et al., 1985; Deng et al., 2001). The umbrella cells, together with subjacent intermediate and basal cell layers, also have a sensory function and in response to various physical and chemical stimuli they can release neurotransmitters (such as ATP and nitric oxide), which then communicate the state of the tissue's external environment to afferent neurons (Apodaca, 2004; Apodaca et al., 2007)

The epithelium of the urogenital tracts of animals is protected from potentially harmful environmental substances by a mucous layer. Glycoproteins of the urothelium are involved in diverse functions such as permeability barrier, cell adhesions (Kreft et al., 2010), intercellular signalling, inhibition or promotion of attachment for bacteria (Bock et al., 1985; Lomberg et al., 1986) as well as resisting shear forces, protecting against digestion of the mucosa by the many proteolytic enzymes present in the urine (N'Dow et al., 2005). Mucin glycoproteins from bladder urothelium have been found in the urine of cows (Deng et al., 2001), mice (Buckley et al., 2000; Muthusamy et al., 2008), pigs (N'Dow et al., 2005), rats (Vinter-Jensen and Ørntoft, 1998) and humans (Bramwell et al., 1983; Bhavanandan et al., 1998; N'Dow et al., 2005; Halim et al., 2012). Recently, the urine glycoprotein profile has been considered as a useful tool to detect novel markers for chronic kidney disease in humans (Vivekanandan-Giri et al., 2011).

Lectins have a specific binding affinity for the sugar residues of glycoconjugates. Owing to their specific affinities to a particular sugar, lectins are useful probes for the intracellular localization of sugar residues (Ihida et al., 1991; Danguy et al., 1994) and for characterizing cellular populations as well as their morpho-functional

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Table 1
Lectin used, their sugar specificities and the inhibitory used in control experiments.

Lectin abbreviation	Source of lectin	Concentration (μg/ml)	Sugar specificity	Inhibitory sugar
MAL II*	*Maackia amurensis*	15	NeuNacα2,3Galβ1,4GlcNAc	NeuNAc lactose
SNA*	*Sambucus nigra*	15	NeuNAcα2,6Gal/GalNAc	NeuNAc lactose
PNA	*Arachis hypogaea*	25	Terminal Galβ1,3GalNAc	β-D-Gal
HPA	*Helix pomatia*	25	Terminal αGalNAc	D-GalNAc
SBA	*Glycine max*	25	Terminal α/βGalNAc	D-GalNAc
DBA*	*Dolichos biflorus*	25	TerminalGalNAcα1,3(LFucα1,2)Galβ1,3/4GlcNAcβ1	D-GalNAc
Con A	*Canavalia ensiformis*	15	Terminal/internal αMan > αGlc	Methyl-α-Man
WGA	*Triticum vulgaris*	25	Terminal/internal βGlcNAc ≫ NeuNAc	D-GlcNAc
GSA II*	*Griffonia simplicifolia*	20	Terminal D-GlcNAc	D-GlcNAc
GSA I-B_4	*Griffonia simplicifolia*	20	Terminal αGal	Methyl-α-Gal
LTA	*Lotus tetragonolobus*	25	Terminal αL-Fuc	α-L-Fuc
UEA I	*Ulex europaeus*	25	Terminal L-Fucα1,2Galβ1,4GlcNAcβ	α-L-Fuc

Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Man, mannose; NeuNAc, *N*-acetyl neuraminic (sialic) acid. All lectins were HRP-conjugated except those marked with *, which were biotinylated lectins.

changes (Spicer and Schulte, 1992; Danguy et al., 1994). Lectins have been also used to evaluate the composition of the oligosaccharides in the bladder urothelium of some mammals such as rat (Vinter-Jensen and Ørntoft, 1998), rabbit (Buckley et al., 2000) and human (Neal et al., 1987), whereas no study has been performed in animal species of veterinary interest. The aim of this study was to investigate the glycoprotein pattern expressed in the bladder urothelium from the donkey (*Equus asinus*) by means of lectin histochemistry. The results of this investigation could provide useful data on the state of health of a species in view of the fact that the donkey species is increasingly used in human medicine (pet therapy, production of hypoallergenic milk) and in agricultural work in those areas in which the use of machines is limited.

Materials and methods

Tissue preparation

Urinary bladders from three donkey stallions in good health status, aged 2.5–4 years, were obtained from a local slaughterhouse. Immediately after collection, fragments from the apex and body of the urinary bladders were fixed in 4% (w/v) phosphate-buffered paraformaldehyde for 24 h at room temperature (RT), dehydrated in an ethanol series, cleared in xylene, and embedded in paraffin wax. Serial sections (5 μm thick) were cut and, after wax removal with xylene and hydration in an ethanol series of descending concentrations, were stained by means of the lectin histochemistry according to Desantis et al. (2007) for glycoconjugate characterization.

Lectin histochemistry

The lectins used are listed in Table 1. The PNA, HPA, SBA, Con A, WGA, GSA I-B_4, LTA, UEA-I lectins were horseradish peroxidase (HRP)-conjugated (Sigma–Aldrich, St. Louis, MO, USA), whereas MAL II, SNA, DBA, GSA II were biotinylated lectins (Vector Laboratories, Burlingame, CA, USA). The dewaxed and rehydrated tissue sections were immersed in 3% hydrogen peroxide for 10 min to suppress the endogenous peroxidase activity. Tissue sections treated with HRP-conjugated lectins were rinsed in 0.05 M Tris–HCl buffered saline (TBS) pH 7.4, and incubated in lectin solution at appropriate dilutions (Table 1) for 1 h at room temperature (RT). After 3 rinses in TBS, the peroxidase activity was visualized by incubation in a solution containing 0.05% 3,3′-diaminobenzidine (DAB) and 0.003% hydrogen peroxide in 0.05 M TBS (pH 7.6) for 10 min at RT. Tissue sections incubated in biotinylated lectins for 1 h at RT (MAL II, SNA, DBA, and GSA II) were rinsed 3 times with 0.05 M phosphate-buffered saline (PBS) and were incubated in streptavidin/peroxidase complex (Vector Labs., Burlingame, CA, USA) for 30 min at RT. After washing in PBS, peroxidase activity was developed in a 3,3′-diaminobenzidine–hydrogen peroxide solution as above. Finally, the sections were counterstained with hematoxylin, dehydrated and mounted.

Controls for lectin staining included: (i) incubation with lectin-free substrate medium, and (ii) incubation with each lectin in the presence of its hapten inhibitory sugar (0.2–0.5 M in Tris buffer) (Sigma–Aldrich, St. Louis, MO, USA) (Table 1).

Enzymatic and chemical treatments

To demonstrate the presence of sialic acids as well as to identify the carbohydrate residues linked to terminal sialic acids, some sections were digested at 37 °C for 16 h with 0.86 U sialidase (Type V, from *Clostridium perfringens*; Sigma–Aldrich) in 0.1 M sodium acetate buffer pH 5.5 containing 10 mM $CaCl_2$ and thereafter subjected to the staining procedures described above with the lectins MAL II, SNA, PNA, HPA, DBA, SBA, WGA. Prior to sialidase digestion the samples were saponified with 0.5% w/v KOH in 70% ethanol for 15 min (Reid et al., 1978) to detach acetyl substituents contained in the acetylated groups on C_4 of the pyranose ring of sialic acid residues, thus rendering the residues susceptible to sialidase digestion (Moschera and Pigman, 1975) and subsequent neuraminic acid cleavage. As controls of the enzymatic digestion, sections were incubated in enzyme-free buffer. Finally, slides were counterstained with hematoxylin.

Slides were observed and photographed under a Leica DMRBE photomicroscope equipped with a Leica DC 300 digital camera and Leica QWin V. 2.3 software for computer-assisted image acquisition and analysis (Leica Microsystems, Wetzlar, Germany). The evaluation of staining intensities was based on subjective, blind estimates of three observers and visually assessed on an arbitrary 3-point scale.

Results

The urothelium lining the donkey urinary bladder is composed of three distinct layers: from the basal zone to the lumen they consist of basal, intermediate and superficial (umbrella) cells. When the bladder is empty as in the specimens used for this study, the bladder mucosa is raised to form large folds with a crypt-like appearance.

The results of lectin staining pattern of the donkey urinary bladder urothelium are summarized in Table 2. No differences were detected, related to the age of the donkeys used in this study.

MAL II (Fig. 1) reacted moderately with the cytoplasm of some intermediate cells and the cytoplasm of all umbrella cells. In addition, the cell surface was stained weakly in the basal cells and strongly in the intermediate cells and umbrella cells. MAL II reactivity was not found in the basal and intermediate layers of the