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RESEARCH ARTICLE

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A histochemical approach to glycan diversity in the urothelium of pig urinary bladder

Maria Mastrodonato[1] | Donatella Mentino[1] | Angela Lopedota[2] | Annalisa Cutrignelli[2] | Giovanni Scillitani[1]

[1]Department of Biology, Laboratory of Histology and Comparative Anatomy, University of Bari Aldo Moro, via Orabona 4/a, Bari, I-70125, Italy

[2]Department of Pharmacy – Drug Sciences, University of Bari Aldo Moro, via Orabona 4/a, Bari, I-70125, Italy

Correspondence
Giovanni Scillitani, Laboratorio di Istologia e Anatomia comparata, Sezione di Biologia Animale e Ambientale, Dipartimento di Biologia, Università degli studi di Bari Aldo Moro, via Orabona 4/a, I-70125 Bari Italy.
E-mail: giovanni.scillitani@uniba.it

Abstract

Intracellular glycans in the urothelium of urinary bladder of 10 adult male Landrace pigs were characterized in situ by immunohistochemical detection of Muc1 mucin by anti MUC1 from rabbit, conventional histochemical techniques (Periodic-Acid Schiff, Alcian Blue pH 2.5, High-Iron Diamine), and binding with 13 lectins (PNA, DBA, RCA-I, WGA, SBA, BSI-B4, ConA, AAA, UEA-I, LTA, LFA, MAA-II, SNA) combined with chemical and enzymatic pre-treatments (β-elimination, desulfation and neuraminidase) to gather reference data for this model animal. Muc1 mucin was detected in the secreting granules of superficial cells and the underlying layer of intermediate cells. The secreting granules in both intermediate cells and superficial cells were rich in carbohydrates, with the oligosaccharidic chains mostly O-linked to proteins. Glycoproteins were prevailing over glycosaminoglycans (GAGs). In both superficial and intermediate cells sulfated and/or sialylated glycans were present, sulfation decreasing in the deeper layers. Lectin-binding detected presence of terminal sialic acid linked mostly in α2,6 to GalNAc, Gal terminal or subterminal to sulfates, GalNAc, GlcNAc, and Fuc, mostly linked in α1,6, α1,3 α1,4 and α1,2 to GlcNAc or Gal, but not to lactosamine chains. Except for fucosylation, the oligosaccharidic chains in the glycoproteins of the urothelium of pig urinary bladder were similar to those linked to human MUC1, which is fundamental in cell adhesion and immunological processes in the urothelium. The co-distribution of Muc1 and saccharidic residues suggests that many of them are linked to the glycoprotein.

KEYWORDS

pig, urinary bladder, mucins, histochemistry, lectins

1 | INTRODUCTION

The urinary bladder is a hollow, musculo-membraneous organ accumulating and excreting urine outward through the urethra. The impermeability of the bladder wall is due to the peculiar properties of the urothelium, i.e., the transitional epithelium lining the urinary tract. The urothelium is made by three layers, each characterized by a distinct cell type. The innermost layer presents basal cells, the intermediate consists of intermediate cells and the apical layer is made by superficial cells, or umbellocytes (e.g., N'Dow et al., 2005). The latter represents the luminal barrier against urine penetration and have distinctive membrane features, such as the presence of tight junctions, thickened apical membrane plaques made by integral proteins known as the uroplakins, and a layer of GAGs and mucins coating the apical surface (Kreft et al., 2005). Epithelial cells in the urothelium differentiate while migrating from the basal to the apical layer, and accumulate intracellular mucins (Brobst et al., 1971).

Mucins from the human urothelium are the most studied and are mainly encoded by the MUC gene family, such as MUC1, MUC4, and MUC3 (N'Dow et al., 2005). Among these, the mucin encoded by MUC1 is one of the most expressed and is a heavy-weight transmembrane glycoprotein rich in serine, threonine and proline with a high amount of O-linked glycans (Bhavanandan et al., 1998). In other mammals a similar mucin is expressed by a MUC1 homologue named Muc1 (Lacunza et al., 2009).

The extra-cellular domain of MUC1/Muc1 is basically made by about 20 aminoacids in tandem repeats (TR), their number being

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variable because of high gene polymorphism. TR domain has multiple potential O-glycosylation sites, and five potential N-glycosylation sites are known in the transmembrane domain (Taylor-Papadimitriou et al., 1999). In non-human species the glycoprotein presents a high amount of conserved sequences in both the transmembrane and cytoplasmic domains, such as the tyrosine phosphorylated cytoplasmic tail (CT), whereas in the TR domain only the sequences in the scaffold are conserved for O-linkage of glycans (Lacunza et al., 2009). The function of the high-conserved sequences is unknown, even it has been suggested that these domains are involved in signalling and protein interactions (Hanisch and Müller, 2000). Compared to the proteic scaffold, the glycan component of MUC1/Muc1 is more variable and less known. Glycosylation patterns can even change from one tissue to another in a same species (Hanisch and Müller, 2000).

The expression of MUC1/Muc1 in several tissues suggests that this glycoprotein plays an important role in the regulation of a number of cellular functions, such as protection and lubrication, signalling, cell proliferation, apoptosis, adhesion and invasion, as well as protection against infectious diseases (Russo et al., 2006; Lacunza et al., 2009).

MUC1/Muc1 expression is known to vary in pathological conditions. As an example, in the normal condition MUC1 is expressed predominantly in the apical membranes of superficial cells, whereas in neoplasies an increase of aberrant forms have been demonstrated by histochemistry, expressed in both the intermediate and in the basal layer of the urothelium (Stojnev et al., 2014). There is considerable evidence that the MUC1/Muc1 mucin interacts with microbial pathogens and mucoadhesive substances, that either require a direct binding to or penetration of the mucosal epithelial cells to cause damage.

From all the above emerges that knowledge about the glycan composition of mucins in the urothelium is very important to understand their role in the regulation of a number of functions, the involvement of their alterations in the insurgence of pathologies, and the precise tuning of therapies, such as intravesical pharmacological treatments (e.g., Lopedota et al., 2016). Anyway, relatively few studies are available about mucin expression and glycosylation in non-human species, including those regarded as model organisms. This is the case of the pig, that is frequently used as a model to investigate the mechanisms and the effects of intravesical treatments with drugs interacting with the mucous layer (e.g., Grabnar et al., 2003; Kerec et al., 2005; Kos et al., 2006; Lopedota et al., 2016).

The present study aimed to characterize the glycans of the secreting granules in both superficial and intermediate cells of the urothelium of the urinary bladder of the pig in normal conditions. We focused on lectin histochemistry combined with chemical and enzymatic treatments and immunohistochemistry to evaluate co-distribution of saccharidic chains and Muc1.

In this way we intended to contribute to the knowledge of the composition of such an important component in bladder functionality and gather data to serve as a possible baseline for further studies to understand its metabolism and the chemical reactions related to the glycan expression, as well as its variations in different pathological and experimental conditions.

2 | MATERIAL AND METHODS

2.1 | Animals and tissue collection and processing

Urinary bladders from ten healthy adult male Landrace pigs, about 1-year old, mean weight kg 101.24 ± 2.93, were obtained from a local slaughterhouse. The urinary bladders, mean weight g 71.12± 4.21 s.d., were rapidly washed in PBS pH 7.4, 0.1 M. Two samples per individual were collected from the bladders body. The samples were fixed with 4% paraformaldehyde in 0.1M saline phosphate buffer (PBS), pH 7.4, for 4 h at 4 °C. After several rinses in PBS, pieces were incubated overnight, at 4 °C, in PBS with 6.8% added sucrose, and dehydrated with increasing acetone, at the same temperature. Embedding was performed by incubating the specimens in a Technovit 8100 (Heraeus-Kulzer, Wehrheim, Germany) glycol methacrylate monomer. Embedding was performed using the Technovit 8100 kit as follows: the infiltration solution was prepared by mixing 100 ml Technovit 8100 basic solution (2-hydroxyethyl methacrylate) with 0.6 g Hardener I (benzoyl peroxide) and was pre-cooled for direct transfer of specimens from acetone. After 6-10 h of incubation at 4 °C the specimens were transferred in the polymerization solution, which was prepared by mixing 30 ml infiltration solution with 1ml Hardener II. (3,5,N,N-tetramethylaniline). Samples were then inserted in polyethylene embedding moulds, which were covered with the polymerization solution and hermetically sealed with cover foils. Hardening was performed on a thin layer of ice at 4 °C for 3 h. Semithin sections (3 μm thick) were cut with glass knives using an LKB Ultratome. Some sections were preliminarily stained with hemalum-eosin (HE) to assess the general morphology and integrity of tissues.

2.2 | Immunohistochemistry

For immunohistochemistry assay, the sections were microwave pretreated in pH 6.0 citrate buffer for 3-5min cycles for antigen retrieval (Gown et al., 1993). The sections were treated with the blocking buffer 1% normal goat serum (Sigma St. Louis, MO) in PBS for 30 min at room temperature and incubated overnight with rabbit anti MUC1 primary antibody (Sigma St. Louis, MO, USA) diluted 1:100 in blocking buffer at 4 °C. After several rinses in PBS, sections were incubated with the secondary antibody, i.e., anti-rabbit Alexa Fluor 488 (Molecular Probes; Eugene, OR, USA) diluted 1:200 for 2 h at room temperature. After several washes in PBS, sections were mounted in a PBS-glycerine 70% mixture for observation.

2.3 | Controls

Negative controls were performed by omitting the primary antibodies or using antibodies preadsorbed with the immunizing peptide.

2.4 | Histochemistry

Sections were stained with Periodic acid-Schiff (PAS), Alcian Blue (AB) at pH 2.5 or High Iron Diamine (HID), and counterstained with hemalum. PAS detects a number of carbohydrates with 1:2 glycol groups

TABLE 1 Lectins used and their carbohydrate specificities

Acronym of lectins	Lectin source	Sugar specificities	Dilution μg/ml	Inhibitory sugar
PNA	*Arachis hypogaea*	Galβ1,3GalNAc	20	Gal
DBA	*Dolichos biflorus*	GalNAc	20	GalNAc
RCA-I	*Ricinus communis*	Gal/GalNAc	10	Gal
WGA	*Triticum vulgaris*	GlcNAc/ Neu5Ac	20	TACT
SBA	*Glycine max*	GalNAc	20	GalNAc
BSI-B4	*Bandeiraea simplicifolia*	α-Gal	20	Gal
ConA	*Canavalia ensiformis*	Man, Glc	20	MαM
AAA	*Aleuria aurantia*	Fucα1-6GlcNAc; Fucα1,3(β1,4) GlcNAc	10	L-Fuc
UEA-I	*Ulex europaeus*	Fucα1-2Gal	20	L-Fuc
LTA	*Tetragonolobus purpureus*	L-Fucα1,6GlcNAc L-Fucα1,2Gal β1,4[L-Fuc1,3] GlcNAcβ1,6R	10	L-Fuc
LFA	*Limax flavus*	Neu5Ac	10	NeuAc / NeuGC
MAA-II	*Maackia amurensis*	Neu5Acα2,3Galβ1,3GalNAc	15	NeuAc
SNA	*Sambucus nigra*	Neu5Acα2,6GalNAc	15	GalNAc

Abbreviations: Fuc, fucose; Gal , galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; MαM, methyl α-mannose Man, mannose; Neu5Ac, neuraminic acid (sialic acid); NeuGC, N-glicolylneuraminic acid; TACT, N,N',N"-triacetylchitotriose.

(Mc Manus, 1948), AB stains acidic glycans (Steedman, 1950) and HID demonstrates acidic, sulfated glycans (Spicer, 1965). Protocol details can be found in Scillitani et al. (2012). Combined staining of AB-PAS or HID-AB were used for the differentiation between acidic and neutral glycoconjugates (Mowry and Winkler, 1956) or to discriminate between carboxylated and sulfated acidic groups (Spicer, 1965), respectively. PAS staining was performed also after β-elimination, a method that removes the O-linked oligosaccharides from glycoproteins (Downs et al., 1973), as follows: sections were incubated with 0.2 M KOH in dimethylsulphoxide–H_2O–ethanol (50:40:10) for 1 h at 45 °C, followed by neutralization with 10 mM HCl and washing in PBS pH 7.4 before staining. All the reagents cited in this section were from Sigma (St. Louis, MO, USA).

2.5 | Lectin histochemistry

Binding of 13 FITC-conjugated lectins (PNA, DBA, RCA-I, WGA, SBA, BSI-B4, ConA, AAA, UEA-I, LTA, LFA, MAA-II, SNA, all from Vector Laboratories, Burlingame, CA, USA), was assessed to characterize the structure and distribution of glycosidic chains. The choice of lectins was made on the basis of the literature about the probable structure of the oligosaccharide chains of mucins in the urinary bladder of mammals (e.g., Desantis et al., 2013 and references therein). The common names, sugar specificities and concentrations of the lectins used are shown in Table 1.

Sections were incubated for 1 h at room temperature with the FITC-lectin solution in Hepes pH 7.5, 10 mM. Sections were subsequently rinsed in the same buffer and mounted in N-propyl gallate in 50% glycerol for observation.

Lectin binding was also tested after desulfation (see below). PNA and DBA staining was performed with and without pre-treatment with neuraminidase, to remove sialic acid (see below). Otherwise specified, all the chemicals cited in this section were from Sigma (ST. Louis, MO, USA).

2.6 | Chemical treatment

Desulfation preceding lectin staining and sialidase digestion was performed by a sequential methylation–saponification. Sections were immersed in 0.15 N HCl in methanol for 5 h at 60 °C and then in potassium hydroxide 1% in 70% ethanol for 15 min at room temperature (Scillitani et al., 2012).

2.7 | Sialidase digestion

Sialidase digestion was carried out by incubating sections with 1 U/ml sialidase from *Clostridium perfringens* (Neuraminidase Type V, Sigma) in 0.1 M acetate buffer pH 5.3 containing 10 mM $CaCl_2$, for 48 h at 37 °C in a moist chamber (Alonso et al., 2003).

2.8 | Control

Controls for lectin-binding were performed by: (1) substitution of the lectin solution with the buffer alone; (2) pre-incubation of the sections with the corresponding hapten sugar inhibitor at a concentration of 0.2 M (Table 1). The effectiveness of the desulfation technique was confirmed by subsequent abolition of the positivity to HID. Controls for enzymatic digestion were performed by the substitution of the enzymes with buffer solution alone under the same incubation conditions to determine the influence of the enzyme-free buffer (Plendl et al., 1989).

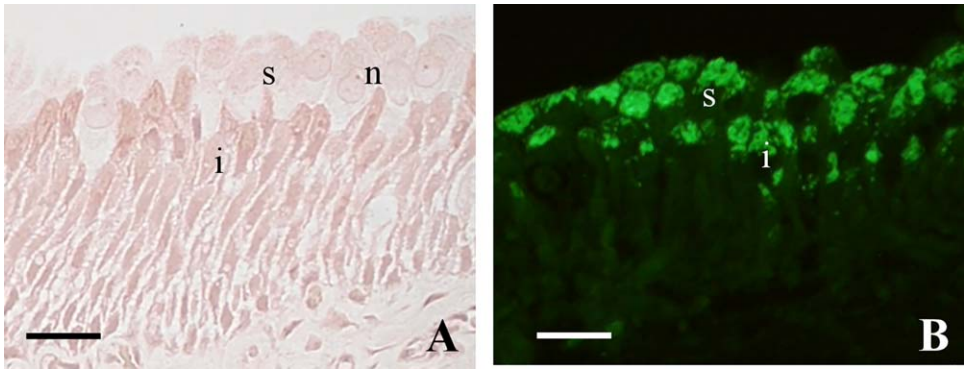

FIGURE 1 Urothelium of pig. **A**: Hemalum-Eosin. General morphology of the urothelium. **B**: Anti MUC1. The secreting granules of superficial cells and the underlying layer of intermediate cells showed the immunoreactivity to the anti MUC1. Bars: 50 μm. Abbreviations: i, intermediate cells; s, superficial cells; n, nucleus [Color figure can be viewed at wileyonlinelibrary.com]

2.9 | Image analysis

The histological images were captured using an Eclipse E600 photomicroscope equipped with epifluorescence and a DMX1200 digital camera (Nikon Instruments SpA, Calenzano, Italy). Histochemical stains were observed under bright light, whereas immunohistochemical staining and lectin-binding were observed in fluorescence under 495 nm light emission. To allow comparisons, the images were taken under the same conditions of bright or fluorescence light intensity. For each stain one to three fields per sample were considered, from which superficial and intermediate cells having the best orientation and staining conditions were selected for the analyses, amounting to about 200 cells per type. Analyses were performed by the ImageJ package (Rasband, 2016) implemented with the colour deconvolution plugin (Landini, 2015).

For fluorescence stains (lectins), the corrected total cell fluorescence (CTFC) for each cell was computed according to McCloy et al. (2014). Integrated density (ID) and area (A) of each cell were measured, then ten regions in the field without fluorescence around the cells were selected as a background and their mean density value (BD) was computed. CTFC was then calculated as $CTFC = ID - (A \times BD)$.

For bright-field histochemical stainings (PAS, AB pH 2.5, HID) RGB images were processed by the colour deconvolution procedure (Ruifrok and Johnston, 2001), which allowed to separate colour channels relative to the stain, the counterstain and the background, respectively. Stain vectors were created from single-stain (i.e., without counterstaining) slides following Landini (2015) procedure. In the stain channel mean intensity was estimated for each selected cell and converted to optical density (OD) by the formula $OD = \log(255/\text{mean intensity})$, which allowed to quantify the "average darkness" due to the staining signal.

Mean CTFC and OD values from each staining underwent statistical analyses to find significant differences among cells and/or methods. Both parametric one-way Analysis of Variance (ANOVA) and non-parametric Kruskal-Wallis test (KW) were performed on means. Post-hoc pairwise comparisons were computed by both parametric Tukey's Honestly-Significant-Difference (THSD, following ANOVA) and non-parametric Dwass-Steel-Chritchlow-Fligner (DSCF, following Kruskal-Wallis) tests. Significance for probability computed from tests was set at $p < 0.01$. Continuous numerical data were then converted to a semi-quantitative evaluation of the intensity of staining by dividing the range of mean values of CTFC or OD into four and three intervals, respectively. Intervals were given progressive numbers and labelled as follows: 1 = weak staining, 2 = moderate staining, 3 = strong staining, 4 = very strong staining. A "0" label was added to indicate negative staining.

Statistical computations were generated by the Real Statistics Resource Pack software (Release 4.3) (Zaiontz, 2015).

3 | RESULTS

In the urothelium of pig both superficial and intermediate cells presented secreting granules in their supranuclear area. The histological staining with HE showed the general morphology of the urothelium (Figure 1A).

3.1 | Immunohistochemistry

The secreting granules of superficial cells and the underlying layer of intermediate cells showed the immunoreactivity to the anti MUC1 (Figure 1B).

3.2 | Histochemistry

The results of the statistical analyses of images and subsequent estimates of staining intensity are resumed in Table 2 and Figure 2. Both ANOVA

TABLE 2 Results of histochemical staining in the superficial and intermediate cells of pig urinary bladder

Stain	Superficial cells	Intermediate cells
PAS	2	3
β-ELIM PAS	1	2
AB pH 2.5	1	2
HID	2	1/2*

0= negative; 1 = weak; 2 = moderate; 3 = strong staining. * = positivity decreased in the deeper layers.

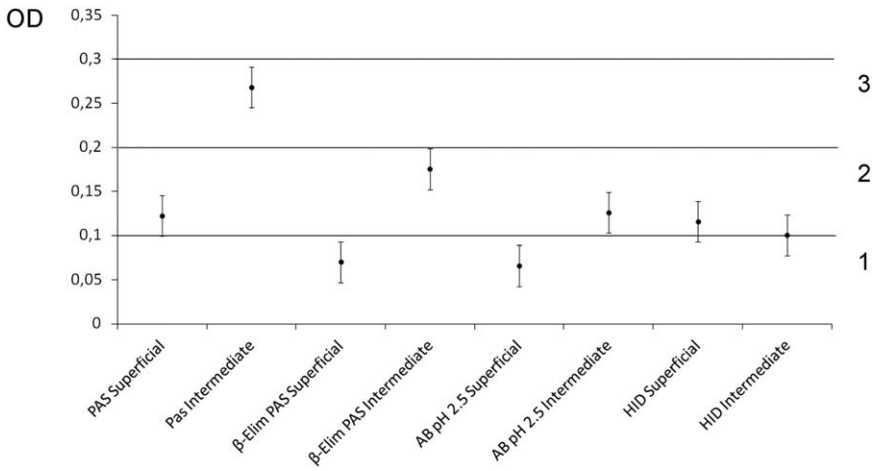

FIGURE 2 Plot of mean OD values and relative standard errors for the histological stains of pig urothelium. On the right there are the numeric labels for each interval in which the range of values was subdivided

and KW tests indicated significant differences among mean ODs (ANOVA: $F_{7,\ 1600\ \text{d.f.}} = 355.268$, $p = 0.000$; KW: H-stat $_{7\ \text{d.f.}} = 1046.472$, $p = 0.000$).

Secreting granules in both cell types were PAS-positive, with a mean intensity resulting moderate in superficial cells and strong in intermediate cells (OD significantly different) (Figure 3A). In both cell types PAS-positivity was significantly reduced after β-elimination to weak (superficial) and moderate (intermediate) (Figure 3B). The secretion of both cell types was stained weak (superficial) or moderate (intermediate) with AB pH 2.5 (Figure 3C). HID stained moderately both the superficial and the immediately underlying layer of intermediate cells, decreasing in the deeper ones (Figure 3D).

Combined AB-PAS (Figure 3E) stained purple most of the granules in the superficial cells, whereas some of them stained blue. Purple staining resulted also in the superficial intermediate cells, whereas the deeper ones stained red.

Combined HID-AB (Figure 3F) stained the granules in the superficial cells blue or brown. In the granules of deeper intermediate cells blue staining predominated over brown.

3.3 | Lectin histochemistry

The results of the statistical analyses of images and subsequent estimates of fluorescence intensity are resumed in Table 3 and Figure 4. Both ANOVA and KW tests indicated significant differences among mean CTFCs (ANOVA: $F_{21,\ 4378\ \text{d.f.}} = 54.589$, $p = 0.000$; KW: H-stat$_{21\ \text{d.f.}} = 968.338$, $p = 0.000$).

3.3.1 | Binding with PNA lectin to detect terminal Gal and to indirectly demonstrate terminal Neu5Ac bound to subterminal Gal

No binding with PNA was observed (upper insert Figure 5A). Desulfation (lower insert Figure 5A) resulted in a weak (superficial cells) to moderate (intermediate cells) and desulfation-sialidase (Figure 5A) pretreatments resulted in very strong (superficial cells) or strong binding (intermediate cells) of the lectin to the secretory granules, significantly higher than desulfation pretreatments alone.

3.3.2 | Binding with RCA-I lectin to detect terminal Gal/GalNAc

RCA-I bound to both superficial and intermediate cells, with the intensity in superficial cells being significantly higher than in the intermediate (Figure 5B).

3.3.3 | Binding with SBA and DBA lectins to detect terminal GalNAc

SBA (Figure 5C) and DBA (Figure 5D) bound to both cell types. Superficial cells showed a significant higher intensity than intermediate with DBA, whereas the opposite was observed with SBA.

3.3.4 | Binding with BSI-B4 lectin to detect terminal Gal

The granules in both superficial and intermediate cells with BSI-B4 (Figure 5) did not bind to lectin.

3.3.5 | Binding with WGA lectin to detect GlcNAc and to indirectly demonstrate terminal Neu5Ac, and binding with SNA, MAA-II and LFA to detect directly Neu5Ac residues

WGA bound moderately to both cell types (Figure 5F). Both SNA and MAA-II bound weak to moderate to both cell types (Figure 6A,B). LFA bound moderately to strong to granules in both cell types and the stained areas appeared larger than those observed with SNA or MAA-II (Figure 6C).

3.3.6 | Binding with ConA lectin to detect Man/Glc residues

ConA linked weakly only to some granules in the intermediate cells (Figure 6D).

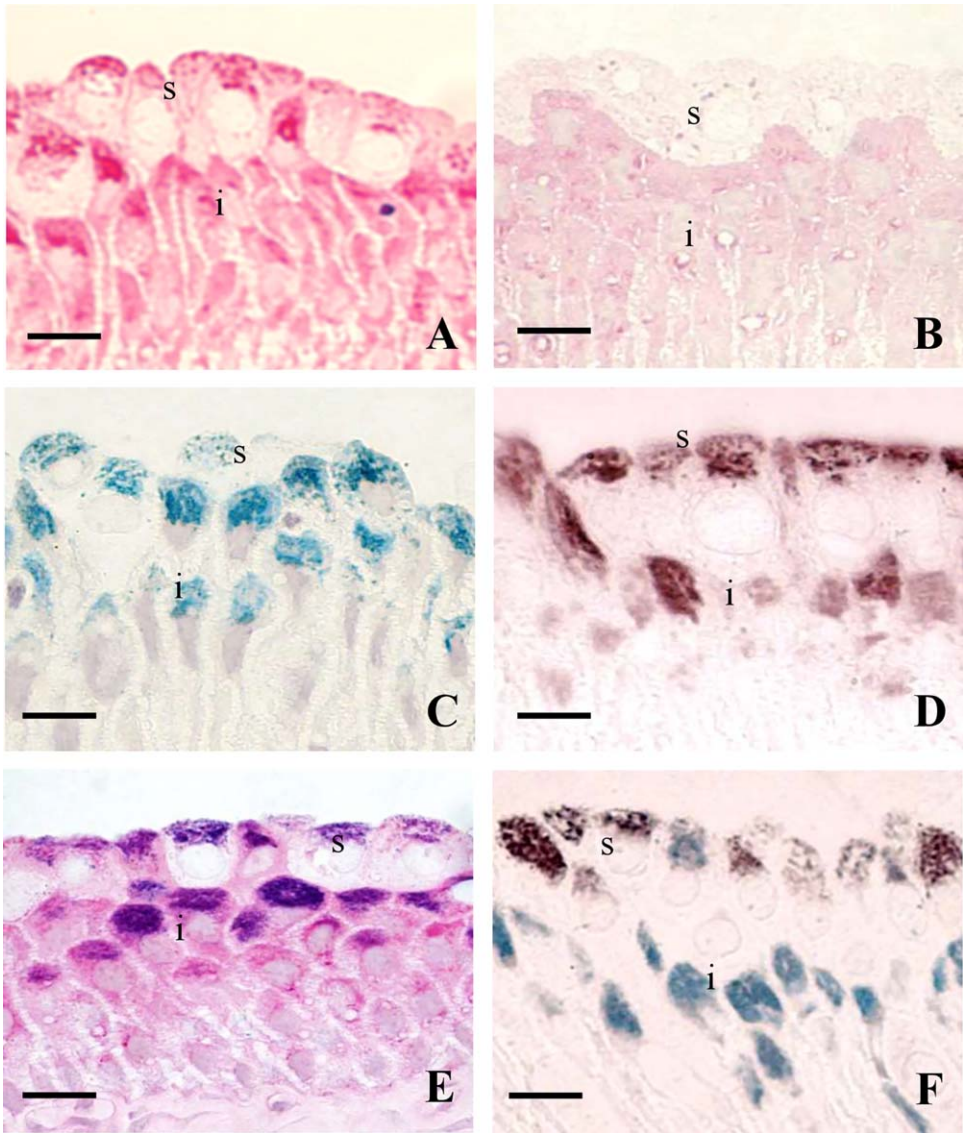

FIGURE 3 Urothelium of pig. **A**: PAS. In both superficial and intermediate cells the secreting granules were PAS-positive. **B**: PAS after β-elimination. The intensity of staining of the secreting granules in both cell types decreased. **C**: AB pH 2.5. The secretion of both cell types stained weak or moderate. **D**: HID. Superficial cells and the most superficial intermediate cells were moderately stained, whereas positivity decreased in the deeper ones. **E**: AB pH 2.5-PAS. Most granules in the superficial cells stained purple, whereas some of them stained blue. Purple staining resulted also in the superficial intermediate cells, whereas the deeper ones stained weakly red. **F**: HID-AB pH 2.5. In superficial cells the secreting granules were stained either brown or blue, or even dark brown because of simultaneous positivity to both staining. Blue stain increased in respect to brown in the intermediate cells granules. Bars: 25 μm. Abbreviations: i, intermediate cells; s, superficial cells [Color figure can be viewed at wileyonlinelibrary.com]

3.3.7 | Binding with AAA, UEA, and LTA lectins to detect Fuc

AAA bound moderately to both superficial and intermediate cells (Figure 6E). UEA-I bound very strongly to the granules of both cell types, but the deeper intermediate cells were negative (Figure 6F). No binding was observed with LTA (not shown).

3.3.8 | Controls

Complete inhibition of lectin labeling resulted from pre-incubation of lectin with the correspondent hapten sugar or omission of lectin from the incubation medium.

4 | DISCUSSION

This work aimed to characterize the glycans diversity of mucins secreted by superficial and intermediate cells in the urothelium of the pig, an animal model for the study of the pathology and the pharmacological treatments of the urinary bladder. Conventional histochemical and lectin techniques combined with chemical and enzymatic treatments allowed an in situ characterization of the oligosaccharidic chains of the secreting granules in superficial and intermediate cells. Immunohistochemical evaluation of expression of Muc1 allowed inferences about co-localization of this mucin and the glycans detected by the

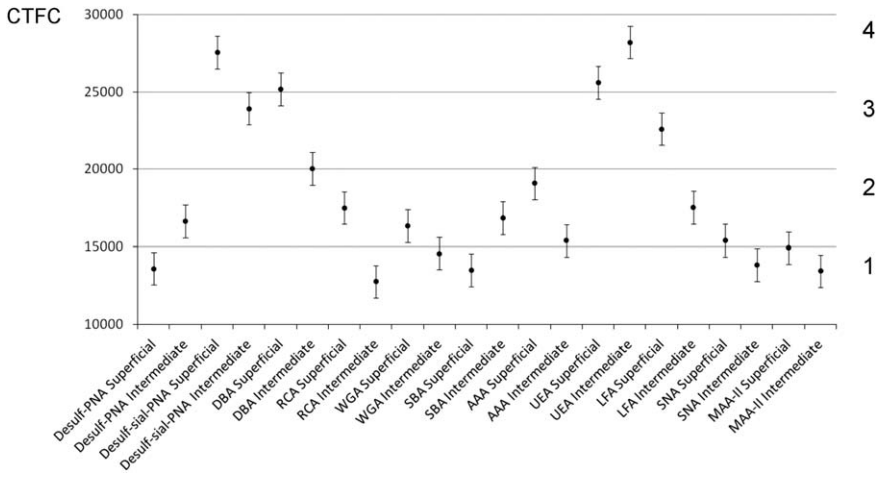

FIGURE 4 Plot of mean CTFC values and relative standard errors for the lectin-binding of pig urothelium. On the right there are the numeric labels for each interval in which the range of values was subdivided

other methods. The histochemical approach is useful in localizing the sites in which glycans are expressed and to evidence differences in both expression and functionality among cells, tissues, organs, developmental stages and species. (e.g., Mastrodonato et al., 2014; Mentino et al., 2014; Scillitani et al., 2012).

Different methods and interpretation of results renders comparison among studies difficult. Traditional evaluation of the intensity of staining in both bright and fluorescent light consists in a semi-quantitative analysis in which observers judge the level of staining by assigning progressive numbers. In an attempt to overcome the subjectivity of such approach, in the present paper staining intensity was estimated by computer-assisted image analysis in which OD (bright light) or CTFC (fluorescence) were computed. It is customary to measure staining intensity in a homogeneous photographic field including several cells, anyway the urothelium structure does not allow this approach because of stratification of cells with varying intensities from the inner to the superficial layers, with not all the cells having an appropriate orientation for measures. Thus, single-cell evaluation from several images captured under same conditions was preferred.

TABLE 3 Lectin binding in the superficial and intermediate cells of pig urinary bladder

Lectin	Superficial cells	Intermediate cells
PNA	0	0
Desulf./PNA	1	2
Desulf/sial/PNA	4	3
DBA	3/4	2/3
RCA	2	1
WGA	2	1
SBA	1	2
BSI-B4	0	0
ConA	0	1*
AAA	2	2
LTA	0	0
UEA-I	4	4
LFA	3	2
SNA	1/2	1
MAA-II	1/2	1

0 = negative; 1 = weak; 2 = moderate; 3 = strong; 4 = very strong staining; *Binding was observed only in few granules.

Several histological stains are not stoichiometric, i.e., there is not a direct relationship between the stain intensity and the amount of reaction products as predicted by the Beer-Lambert law (Landini, 2015). Nonetheless, intensity values can be useful for comparative purposes, as in the present paper.

In the urothelium of the pig bladder the Muc1 mucin resulted expressed mainly in the apical surface of superficial cells and in the immediately underlying lines of intermediate cells. A number of high conserved sequences shared with human MUC1 allows the use of antibodies against sequences from MUC1 to detect and characterize Muc1 in other species (Lacunza et al., 2009), as we did.

The secreting granules in both superficial and intermediate cells were rich in carbohydrates, as evidenced by PAS staining. The heavy reduction of PAS positivity after β-elimination indicates that the oligosaccharidic chains are mostly O-linked to proteins. Positivity to AB pH 2.5 indicated the presence of acidic glycans. In several cells AB-PAS co-staining resulted in a purple stain, indicating the co-presence of neutral and acidic residuals in glycoproteins, whereas a little number of cells were stained blue only, probably due to GAGs. This suggests that glycoproteins prevail over GAGs, as it has been observed in other mammals (Rubben et al., 1983; Bhavanandan and Erickson, 1997; Buckley et al., 2000; Pereira et al., 2004). Co-staining with HID-AB resulted in both blue and dark-brown cells, indicating co-presence of acidic glycans sialylated or sulfated, respectively. The increase of HID intensity from the deeper interstitial cells to superficial cells could be explained by a progressive sulfation of mucins in the course of the

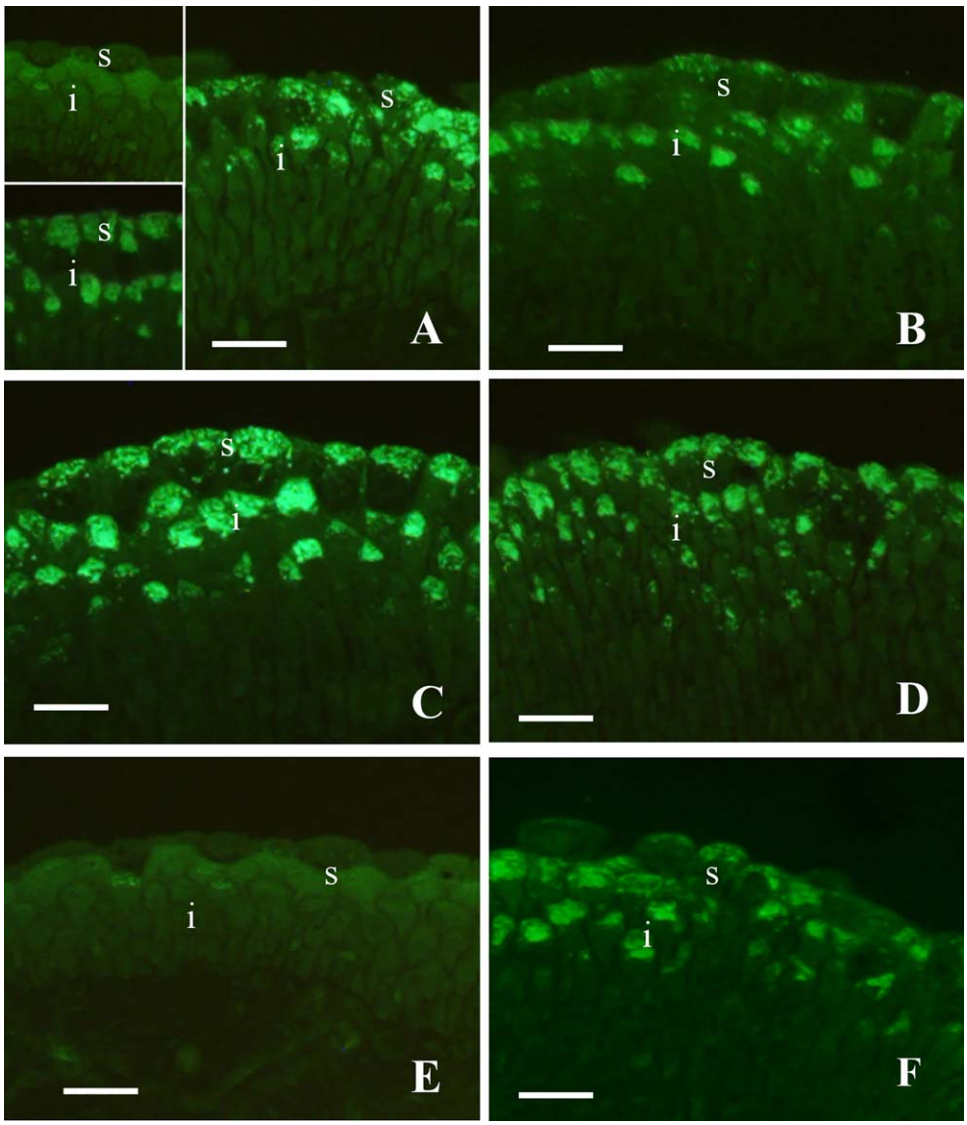

FIGURE 5 Urothelium of pig. **A**: Binding with PNA lectin (FITC-conjugated). No binding with PNA was observed (upper left), PNA binding increased after desulfation (lower left) and the increase was more evident after desulfation-sialidase digestion (right). **B**: Binding with RCA-I lectin (FITC-conjugated). Both superficial and intermediate cells were bound. **C**: Binding with SBA lectin (FITC-conjugated). The lectin bound to both cell types. **D**: Binding with DBA lectin (FITC-conjugated). Moderate to strong binding of the lectin to the granules in both superficial and intermediate cells was observed. **E**: Binding with BSI-B4 lectin (FITC-conjugated). Both cell types did not shown affinity for this lectin. **F**: Binding with WGA lectin (FITC-conjugated). WGA bound weak to moderate to both cell types. Bars: 25 μm. Abbreviations: i, intermediate cells; s, superficial cells [Color figure can be viewed at wileyonlinelibrary.com]

migration of cells from the basal to the apical layer. Sulfated and sialylated glycans confer a strong negative charge to the apical surface of the urothelium. This constitutes a barrier against the penetration of anions and influences the rheological features of mucus as well as cell interactions. Among GAGs, condroithin sulfate is localized on the apical surface and its removal increases the permeability of membranes (Janssen et al, 2013). Besides, sulfates inhibit inflammatory processes and bacterial mucin degrading glycosidases (Kawashima, 2006). PNA linking only after desulfation confirmed the presence of sulfates, since they can prevent lectin binding (Parillo et al., 2000). Besides, it indicated that sulfates are added to Gal when it is β1,3-linked to GalNAc, whereas RCA-I binding without desulfation suggests that most sulfates are not linked to Gal in lactosamine dimers (Galβ1,4-GlcNAc). Apart from linking sulfates, Gal is involved in intercellular recognition, as demonstrated by the presence of two galectins (i.e., endogenous Gal-binding lectins) expressed by the apical surface of the urothelium in the pig, rabbit, mouse and human (Bhavanandan et al., 2001). Finally, galactosylated residuals in the rabbit inhibit the adhesion of the pathogenic bacterium *Pseudomonas aeruginosa* (Chiarini et al., 1990). The presence of carboxylated glycans was indicated by the blue-stained granules detected by combined AB pH 2.5-HID. LFA-binding demonstrated that carboxylation is due to sialic acid in terminal position in the saccharidic chains, whereas SNA and MAA-II binding cleared the patterns of linkage, indicating α2,6 binding to GalNAc or α2,3 binding to

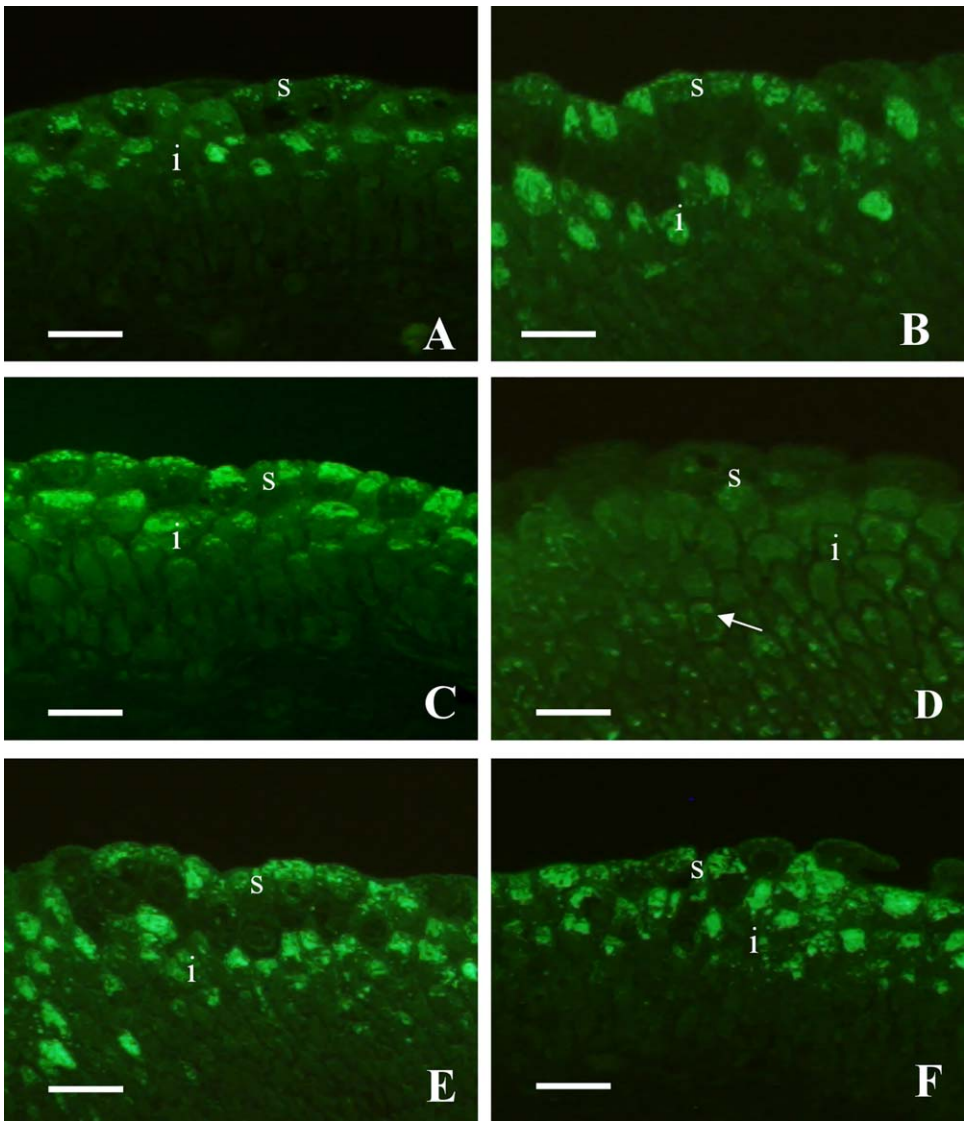

FIGURE 6 Urothelium of pig. **A-C**: Binding with SNA, MAA-II, LFA lectins (FITC-conjugated). Both superficial and intermediate cells were weak to moderate bound to SNA (A) and MAA-II (B). The granules in both cell types bound stronger with LFA lectin than with either SNA or MAA-II (C). **D**: Binding with ConA lectin (FITC-conjugated). Both cell did not shown affinity for this lectin although some granules in the intermediate cells showed a weak binding (arrow). **E**: Binding with AAA lectin (FITC-conjugated). This lectin bound moderately to both superficial and intermediate cells. **F**: Binding with UEA lectin (FITC-conjugated). UEA-I bound very strongly to the granules of both cell types, but the deeper intermediate cells were negative. Bars: 25 μm. Abbreviations: i, intermediate cells; s, superficial cells [Color figure can be viewed at wileyonlinelibrary.com]

Galβ1,3GalNAc, respectively. Sialoglycoproteins were observed in the urothelium of human, rabbit, rat and donkey (Amano et al., 1991; Bhavanandan and Erickson, 1997; Desantis et al., 2013). Sialic polyanionic chains act in a way similar to sulfates in regulating the aforesaid processes. Variations in the expression of sialic acid are known in a number of pathological conditions of human urinary bladder (Videira et al., 2009). Besides, sialic acid mediates the interactions between pathogens and urothelial cells: in some cases, the high negative charge of sialylated chains prevents bacterial adhesion (Callahan et al., 1985), whereas in others sialic acid is involved in recognition and adhesion (Sakarya et al., 2003; Anderson et al., 2010).

The presence of GlcNAc was detected by WGA-binding. GlcNAc is particularly important in relation to acidification of mucins. In fact, sulfate groups are linked to Gal or GlcNAc in the oligosaccharide chains. Fucose is widespread in O- and N-linked glycans and in glycolipids of mammalian cells and is usually terminal in the saccharidic chains (Becker and Lowe, 2003). Its presence in both intermediate cells and superficial cells was detected by AAA-, UEA-I and LTA-lectin binding. AAA-lectin binding detects the presence of Fuc α1,6-linked to core GlcNAc in N-liked glycans and terminal Fuc α1,3 or α1,4-linked to GlcNAc in O-linked glycans (Yamashita et al., 1985; Osawa and Tsuji, 1987; Spicer and Schulte, 1992). An amount of Fuc is also α1,2-linked

to Gal, as indicated by UEA-I binding. Negative results for LTA-binding suggest the lack of Fuc linked to lactosamine chains. Fucose expression detected by LTA-binding is observed in bladder carcinome (Shirahama et al., 1993). Fucose is important in determining rheological properties of mucus, anyway in other mammalian species only small amounts are expressed (Desantis et al., 2013). Thus, the condition of the pig is unusual since Fuc is highly expressed in the normal condition. Anyway, LTA-detected Fuc residuals like those expressed in bladder carcinoma were not found.

DBA- and SBA-lectin binding indicates the presence of GalNAc. It is an important core residual linking the chain to serine or threonine in the protein backbone (Chaturvedi et al., 2008). GalNAc is also a fundamental component of chondroitin sulfate, forming tandem-repeated dimers with glucuronic or iduronic acids (Kamhi et al., 2013). Thus, lectin binding could be partly due to the presence of chondroitin sulfate, in which GalNAc contributes to the structure and functions of these GAGs.

In conclusion, the results of our study evidenced in the urothelium of pig urinary bladder the presence of glycoproteins and a lesser amount of glycosaminoglycans. The oligosaccharidic chains in the glycoproteins were similar to those of human MUC1, in which the main chains are NeuAcα2,6GalNAc, NeuAcα2,3Galβ1,3GalNAc and (Galβ1,4GlcNAcβ1,6)(NeuAcα2,3Galβ1,3) GalNAc (Hanisch and Müller, 2000). Also the distribution of the Muc1 in the pig urothelium somewhat parallels that of human, in which MUC1 is localized in the apical part of the urothelium of urinary bladder (Russo et al., 2006). The co-distribution of anti MUC1 and lectin-binding residuals in superficial cells and neighboring intermediate cells suggests that the detected glycans may be linked to the Muc1 scaffold.

Our study on normal pig urinary bladder contributes to the knowledge of the mucins composition essential in bladder functionality and it can be a useful tool to evaluate alterations of urothelial mucins under different physiological, pathological or experimental conditions, with possible translational value in human. Caution should be paid to the higher fucosylation observed in respect to other species.

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