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Glycopattern analysis of acidic secretion in the intestine of the red-eared slender turtle; *Trachemys scripta elegans* (Testudines: Emydidae)

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ABSTRACT

The secretion of the goblet cells in the intestine of *Trachemys scripta elegans* was studied in situ by histochemical methods to analyze the diversity of sugar chains, with particular regard to the acidic glycans. Conventional histochemical stains (Periodic acid-Schiff, Alcian Blue pH 2.5, High Iron Diamine) and binding with ten FITC-labelled lectins combined with chemical and enzymatic pre-treatments were used to characterize the oligosaccharidic chains. The intestine can be divided into three regions, i.e. a duodenum, a small intestine and a large intestine. Goblet cells were observed in all the three tracts and presented an acidic secretion. WGA, LFA, PNA and SBA binding was observed only after desulfation. Glycans secreted by the three tracts consist mainly of sulfosialomucins with 1,2-linked fucose, mannosylated, glucosaminylated and subterminal galactosyl/galactosaminylated residuals. Differences among tracts are quantitative rather than qualitative, with sulfated, galactosaminylated and glycosaminylated residuals increasing from duodenum to large intestine, and galactosylated and fucosylated residuals showing an opposite trend. Variation is observed also between apices and bases of villi in both duodenum and small intestine, where sulphation decreases from the base to the apex and glycosylation shows an opposite trend. Functional implication of these findings is discussed in a comparative context.

1. Introduction

From an anatomical point of view, the intestine of living non-avian Sauropsida (i.e., turtles, crocodiles, tuataras, lizards and snakes, hereafter referred as NAS) is usually regarded as being less complicated than that of birds and mammals (e.g., Stevens and Hume, 2004). Apart being relatively shorter, it lacks some differentiated secreting areas such as the Brunner's glands or true Lieberkühn crypts (e.g., Luppa, 1977). By contrast, the diversity of composition of the mucins produced by the intestinal glandular cells of the NAS as revealed by lectin histochemistry is comparable to those of birds and mammals (e.g., Schumacher et al., 1986; Perez-Tomas et al., 1990; Sharma and Schumacher, 1992; Scillitani et al., 2012). In a same species, mucin variation among tracts is linked to different physiological requirements, such as protection against gastric acid, pathogens, mechanical damages, regulation of food and water absorption, and interaction with the bacterial flora (e.g., Corfield et al., 2001; Becker and Lowe, 2003; Robbe et al., 2004). Among species, variation of mucin composition in a same tract is known (e.g., Schumacher et al., 1986) and can be explained both in term of different functionality (e.g., Awaad, 2016) or phylogenetic history (e.g., Gabe and Saint Girons, 1972).

Intestinal acidic glycans are of particular interest, since they are involved in several of the already cited functions. Sialic acids are among the most important residues in these glycoconjugates (e.g., Varki and Schauer, 2009). Being electronegatively charged, they can stabilize the glycoprotein conformation, influence the rheological properties of the mucus and mediate the adsorbing processes. They are often in terminal position in the saccharidic chains, which allows them to interact with other cells and pathogens, preventing their attack but also acting as recognition sites for several receptors, toxins and microorganisms (Schauer, 2007). Besides, sialome modifications are known to occur in several pathologies (e.g., Corfield et al., 2001). Acidic glycans can be also sulfated. Similar to the sialylated, the sulfated residuals can affect mucus viscosity. They have also cytoprotective effects against glycosidases and prevent

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bacterial motility and inflammatory disorders (Tobey et al., 1986; Kawashima, 2006; Tobisawa et al., 2010).

Comparative studies among different species attempted to correlate mucin composition to diet. Among NAS, the most extensive dataset available is that of Gabe and Saint Girons (1972) about duodenal secretion in lizards. They found that the acidic content of mucins secreted by the goblet cells in many cases was similar in species belonging to the same family, even if they had different feeding habits, whereas differed among species of distinct lineages with similar diets. Thus, they concluded that mucin composition is affected by phylogenetic affinity rather than alimentation. Data about histochemistry of intestinal mucosubstances in Testudines are fragmentary, since few species were studied by conventional and/or lectin-binding techniques (Madrid et al., 1989; Perez-Tomas et al., 1990; Schumacher et al., 1986; Sharma and Schumacher, 1992; Suganuma et al., 1981). A general picture emerges of an intestinal secretion rich in sialo- and sulfomucins, with sulfation increasing from small to large intestine and oligosaccharidic chains differing in carbohydrate composition among tracts.

Keeping all the previous as reference, we studied the distribution and composition of intestinal mucins by conventional histochemistry and lectin-binding techniques in the red-eared slender turtle, *Trachemys scripta elegans*, of which we previously investigated the distribution of glycoconjugates in the secreting epithelium of the oesophagogastric tract (Scillitani et al., 2008). This species is regarded as a model animal and has omnivorous feeding habits (e.g., Aresco, 2010), thus it would be expected that the morphofunctional features of its intestine somewhat reflect the dietary requirements. Our goals were to detect regional differences in the composition and distribution of mucins, with particular regard to the acidic ones, to estimate the level of diversification in comparison to other NAS and mammals, as well as individuating possible implications of variation in this model reptile.

2. Material and methods

Six feral adult turtles (three females, three males) were caught in an artificial pond near Bari (Italy). Following anesthesia with ether/oxygen, animals were sacrificed by cervical dislocation. Animal treatment followed the EU Directive 2010/63/EU for animal experiments and the ethical standards for experimental protocols approved by the University of Bari. The intestine was rapidly removed and washed in Tris-buffered saline (TBS) pH 7.4, 0.1 M. Samples of about 10 mm were cut and fixed in 10% neutral buffered formalin, dehydrated in a graded series of ethanol, than embedded in paraffin wax. Sections were serially cut at 5 μm.

2.1. Conventional histochemistry

Samples were stained with Periodic acid-Schiff (PAS), Alcian Blue (AB) at pH 2.5 and High Iron Diamine (HID) to demonstrate carbohydrates with 1,2 glycols, acidic residuals (both sialylated and sulphated), and sulphated residuals, respectively. Counterstaining with Mayer's hematoxylin (HE) was performed. Combining AB with PAS or HID in a same section allowed to discriminate between residuals: AB-PAS (Mowry and Winkler, 1956) individuated neutral (stained red) and acidic (stained blue/violet) residuals, whereas HID-AB (Spicer et al., 1965) demonstrated the simultaneous distribution of sialylated (stained blue) and sulfated (stained brown) residuals. For protocol details readers are referred to Scillitani et al. (2012). All the chemicals cited in this section were from Sigma (St. Louis, MO, USA).

Photographic documentation was shot in bright light by an Eclipse E600 photomicroscope equipped with a DMX1200 digital camera (Nikon Instruments SpA, Calenzano, Italy) under the same conditions for all the stains, tracts and samples. Comparison among different tracts of the intestine were performed by computing optical density (OD) values from RGB photographs processed by the colour deconvolution method (Ruifrok and Johnston, 2001). For each stain and tract, one to three fields were selected per sample and goblet cells having the best orientation were chosen for analysis, for a total amount of about 220 cells. Colour deconvolution allowed to separate colour channels relative to the stain, the counterstain (or second stain in combined stain experiments) and the background. Stain vectors were created from single-stain without counterstaining slides (Landini, 2004). Single cell ODs were then computed from mean intensities in each stain channel (see Mastrodonato et al., 2017, for details). Analyses were performed by the ImageJ package (Rasband, 2016) implemented with the colour deconvolution plugin (Landini, 2004). Mean OD values from each staining and tract were compared by parametric one-way Analysis of Variance (ANOVA) and non-parametric Kruskal-Wallis test (KW) to find significant differences. 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 semiquantitative scale by dividing the range of mean OD values into 3 intervals: 1 = weak staining, 2 = moderate staining, 3 = strong staining. Negative staining was indicated by "0".

2.2. Lectin histochemistry

A panel of 9 FITC-labelled lectins (WGA, LFA, PNA, RCA-I, SBA, ConA, AAA, UEA-I, LTA) was selected among the most commonly used to detect the main residuals in the oligosaccharidic chains of mucins. Details for the lectins employed, their concentrations, their sugar specificities and the abbreviations used for glucidic residues are summarized in Table 1. All lectins were from Vector Laboratories (Burlingame, CA, USA). Lectin protocols followed dealer's indications and Scillitani et al. (2011). Sections underwent incubation for 1 h at room temperature with the lectin solution in HEPES and after rinsing in the same buffer were mounted in Fluoromount (Sigma-Aldrich) for observation. Controls for lectin labelling included: 1) substitution of each lectin with HEPES alone; 2) incubation with lectin added to an inhibitory sugar (types and concentrations given in Table 1); 3) binding to the secreting epithelia of duodenum of the mouse or the Guinea-pig, as well as to the egg extra-cellular matrix of the toad *Bufo bufo*, the mucins of which were previously demonstrated to be labelled by the tested lectins (Mentino et al., 2014; Scillitani and Mentino, 2015).

Since negative results were obtained with WGA, LFA, PNA and SBA, the binding of these lectins was also tested with a desulfation pre-treatment by a sequence of methylation-saponification (Spicer and Lillie, 1959). Sections were treated with 0.15 N HCl in methanol at 60 °C for 5 h, then immersed in 1% KOH in 70% ethanol for 15 min at room temperature. To detect the subterminal residues of sialic acid, PNA and SBA-binding were also tested after sialidase digestion following desulfation. Sections were incubated with 1 U/ml sialidase from *Clostridium perfrigens* (Neuraminidase Type V, Sigma-Aldrich) for 48 h at 37 °C, in a moist chamber, in 0.1 M acetate buffer pH 5.3, containing 10 mM $CaCl_2$. The influence of the buffer alone in the digestion reaction was tested by incubating control sections in the same conditions without adding the enzyme (Plendl et al., 1989).

Stable class-III mucins as defined by Katsuyama and Spicer (1978) were detected by the paradoxical ConA binding (PCS): sections were treated with 1% periodate, followed by 0.2% sodium borohydrate in 1% sodium biphosphate before ConA labelling. Bind-

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