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Glycohistochemical study of endogenous lectins in androgen-deprived mice ventral prostate treated by Testosterone and Dihydrotestosterone

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Abstract

We investigated histochemically the expression of endogenous sugar-binding proteins (endolectins) in the ventral prostate of normal mice, castrated mice; castrated mice injected with testosterone (T) and castrated mice injected with dihydrotestosterone (DHT). The avidin-biotin-peroxidase complex technique was used with a panel of biotinylated neoglycoproteins, on paraffin sections. Characteristic binding patterns depending upon the type of neoglycoprotein were observed in the glandular epithelium and basement membranes. This report also initiates the histochemical study of endogenous glycan-binding proteins in this male accessory sex organ. Remarkably, the endocrine status was found to affect the distribution of endolectins in the epithelial cells. Indeed, in the cytoplasm and apical region of the epithelial cells, the androgen deprivation activates the expression of all endolectins;in the basement membrane, the receptors of heparin, melibiose and mannose are activated. The injection of the T to the castrated mice inhibits the receptors for the N-acetylated and fucosylated sugars in the cytoplasm.The DHT suppresses the endogenous lectins with specificities for xylosides, glucosides, galactosides and mannosyl in the apical area. In the basal membrane, no suppression of endolectine is observed following injection of the T or DHT.The histophysiological significance of these modifications in the glandular cells is discussed in the light of previous biochemical findings obtained mainly in humans and the rat.

Keywords: Ventral prostate, Neoglycoproteins, Glycohistochemistry, Testosterone, Dihydrotestosterone.

Introduction

The prostate is an exocrine gland in the male reproductive tract that secretes seminal fluids and plays important roles in the reproductive process. Although the prostate can be affected by several disorders, including, benign prostate hyperplasia and cancer, physiology, pathology and responsiveness of the prostate against an array of hormones have been well studied (Cooper *et al.*, 1999; Hayward & Cunha, 2000). The mouse prostate gland is comprised 4 lobes, namely the anterior, dorsal, lateral, and ventral lobes (Bradshaw & Wolfe, 1977; Samuel & Flickinger, 1986). These lobes differ in terms of their histological examinations, ductal patterns, and secretory functions (Sugimura *et al.*, 1986; Fujimoto *et al.*, 2006). Epithelial cells lining the prostatic lumen are primarily composed of luminal cells and basal cells. The function of luminal cells is to generate various secretory products that are characteristically rich in glycoconjugates (Tsukise & Yamada, 1987), while the function of basal cells remains to be determined.

Glycosylation is one of the most common co- or posttranslational modifications. Inside a cell, complex glycosylation pathways assemble these oligosaccharides and attach them to proteins and lipids as they travel to the cell surface (Campbell *et al.*, 2007).

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Furthermore, by virtue of their peripheral location, particular oligosaccharide epitopes on proteins or lipids exert key functions in important intercellular communication processes such as fertilization (Velásquez *et al.*, 2007), immune response, pathogen anchoring, or metastasis (Jiménez-Castells *et al.*, 2008). These particular carbohydrate epitopes are recognized by membrane-anchored carbohydrate-recognition domains of different molecules such as receptors, enzymes, antibodies, or neoglycoproteins (Jiménez-Castells *et al.*, 2008).

Neoglycoproteins (synthetic conjugates of glycan derivative and its carrier proteins) presenting the chemoenzymatically produced glycans (Unverzagt *et al.*, 2002; André *et al.*, 2004, 2006, 2007). Obviously, neoglycoproteins are versatile tools to study interactions of defined glycans presented by a natural scaffold with sugar receptors. However, endogenous receptors to the cellular glycoconjugates that complete a recognitive interaction within a sugar code system of biological information have only recently attracted experimental attention. By application of synthetic neoglycoproteins as custom-made ligands to this class of proteins, the expression of endogenous sugar receptors has been systematically evaluated with a focus on histopathology (Gabius, 1988). By the use of labelled neoglycoproteins it is feasible to demonstrate histochemically and cytochemically a pattern of natural receptors for common carbohydrate constituents of cellular glycoconjugates of which some supposedly have crucial functions in glycophysiology.

The purpose of this study was to provide initial information on both glycobiological aspects of the ventral prostate glands of normal as well as of castrated mice, castrated mice injected with testosterone (T) and castrated mice injected with dihydrotestosterone (DHT). To this end we used a panel of biotinylated neoglycoproteins on Bouin-fixed paraffin-embedded tissue sections (Akif *et al.*, 1993, 1994, 1995; Akif & Genten, 2015).

Materials and methods

Animals and tissue preparation

Sexually mature NMRI mice (8 weeks old) were castred under sodium pentobarbital anesthesia, and used for experiments eight weeks later. Ten castrated males are subject to hormonal administration for five days, each mice received 0.1 ml of hormone solution subcutaneously. Five males were treated with testosterone (T: 4-androsten-17β-ol-3-one) in an amount of 5µg/g, five males received 1µg/g of dihydrotestosterone (DHT: 5α-androstan-17β-ol-3-one). T and DHT are prepared by dissolving them in ether adding the appropriate volume of corn oil. Before injection, the ether-oil-hormone solution is under the hood to evaporate the ether. The controls were: both castrated and normal males injected with oil only. Mice were sacrificed 24 hours after the last injection. Control mice (sham operated) were used at the same age. Mice were maintained on a regular photoperiod of 12 hour light-12 hour dark at 23°C. Animals were anaesthetized with ether. The accessory sex glands were exposed and bathed with the fixative during dissection. The prostates were removed, fixed in Bouin's solution, embedded in paraffin and sectioned at the thickness of 5µm. After dewaxing, neoglycoprotein histochemical staining was performed using the avidin-biotin method, following optimized procedures (Akif *et al.,* 1993, 1994, 1995).

Neoglycoprotein histochemistry

For detection of the pattern of endogenous sugar receptors with different carbohydrate specificities, the following biotinylated neoglycoproteins (chemically glycosylated derivatives of bovine serum alburnin; BSA) were used. For α-galactoside-specific receptors: Melibiose-

BSA-biotin; N-acetyl-D-glucosamine-(BSA-biotin) and N-acetyl-D-galactosarnine-(BSA-biotin) for receptors with respective specificity for these two naturally occuning N-acetylated sugars; for β-xylosidespecific receptors: β-D-xylose-(BSA-biotin); for mannoside- and fucoside-specific receptors: D-mannose-(BSA-biotin) and L-fucose-(BSA-biotin), respectively; for receptors specific for sugars that contain a phosphate group: D-mannose-6-phosphate-(BSA-biotin). The sulphated glycosaminoglycan, heparin, was also employed after mild cyanogen bromide activation, aminoalkylation and biotinylation. The batch of BSA, used for neoglycoprotein synthesis, had additionally been treated with periodate to destroy any traceable contamination with carbohydrates. Chemical preparation, quality controls and properties of labelled carbohydrate-protein conjugates are described elsewhere (Gabius & Bardosi, 1991).

Rehydrated sections of Bouin-fixed, paraffin-embedded tissues were incubated for 30 minutes in 30 % alcohol containing 0.1 % hydrogen peroxide to block endogenous peroxidase activity, rinsed in phosphate-buffered saline, pH7.4 (PBS), then incubated for 60 minutes with biotinylated neoglycoproteins at a concentration of 10 µg/ml in PBS (100 µg/ml for lactosylated albumin). After thorough rinsing in PBS the sections were incubated for 60 minutes with peroxidase-conjugated avidin (Camon, Wiesbaden, F.R.G.), then rinsed again in PBS. Peroxidase enzyme, localizing bound biotinylated neoglycoproteins, was demonstrated in the tissues by development in 0.5 % diaminobenzidine in the presence of 0.01 % hydrogen peroxide. Sections were counterstained with haematoxylin, dehydrated in graded alcohols and mounted in DPX. Control sections were incubated with biotinylated BSA that had not been chemically modified by glycosylation at a concentration of up to 100 µg/ml to exclude any nonspecific interaction of the unmodified carrier protein with the section. Further control sections to prove specificity of protein-carbohydrate interactions were incubated for one hour with homologous unlabelled (neo) glycoproteins at concentrations between 3µg/ml and 3 mg/ml; these were pipetted off without rinsing and solutions containing 1:1 mixtures of biotinylated and unlabelled (neo) glycoprotein were added, with further incubation for 30 minutes.

Further control experiments were devised to permit distinction between different possible receptor types for neoglycoproteins: (a) to assess or exclude the contribution of glycosidases to staining, incubation of the derivative was performed at low pH values where glycolytic activity is pronounced. The hydrolysis of the carbohydrate from the carrier should greatly diminish binding. Since binding is dependent upon pH, the decrease however could also be due to reduced binding, not compensated by longer incubation times. The results should thus be interpreted with caution.(b) Since glycosyltransferases can be inhibited in histochemistry by the addition of nucleotides (Straus & Keller, 1986), inhibition of the extent of staining in the presence of 20 mm cytidine 5'-diphosphate (CDP), uridine 5'-diphosphate (UDP) and guanosine 5'-triphosphate (GTP) is a measure of the involvement of these enzymes in neoglycoprotein binding.

Results

The neoglycoprotein-binding capacity of the alveolar epithelial cells in normal, castrated mice, castrated mice injected with testosterone (T) and castrated mice injected with dihydrotestosterone (DHT) is summarized in Table 1.

Table 1: Semiquantitatively-determinined intensity of neoglycoprotein and Heparin binding to the mouse ventral prostate epithelium.

Type of neoglycoprotein and Heparin	Normal males				Castrated males				Castrated males +5µg/g testosterone				Castrated males + 1µg/g dihydrotestosterone			
	AR	C	BM	%	AR	C	BM	%	AR	C	BM	%	AR	C	BM	%
Heparin	**0**	0(2)	0	0	3	3(2)	1	50	1	1(2)	1	0	0	0(2)	0	0
Xylose-BSA	2	1	2	100	3	2/3	2	80	2	1	2	100	1	1	2	100
Fucose-BSA	1	1	1	100	2	1/2	1	30	0	0	2	100	1	1	1	100
GalNaAc-BSA	0/1	0/1	1	100	1	1/2	1	80	0	0	1	100	0	0	2	100
GlcNAc-BSA	1	1	1	100	1/2	2	1	80	0	0	1	100	0	0	1	100
Melibiose-BSA	0/1	0/1	0/1	100	1/2	2	1	20	0/1	0/1	0/1	100	0/1	0/1	1/2	100
Mannose-6-P-BSA	1	1	1	100	2	2	2	30	1	1	1	100	1	1	1	100
Mannose-BSA	2	1	1	100	2	2/3	1	80	2	1	1/2	100	1	1	1	100

BSA, bovine serum albumin; **GlcNAc**, N-acetylglucosamine, **GalNAc**, N-acetylglactosamine, **AR**, apical region (including microvilli); **C**, cytoplasm; **BM**, basement membrane; **%**, percentage of labeled cell; **( )**, nuclear labelling; **n/n**, variable reactivity in the same structure. Numbers indicate intensity on an estimated scale from 0 (unreactive) to 3 (strongly reative).

Apical region

The expression of endolectins at this level is maximum in castrated males. Note, however, that the specific receptors of mannose are the exception; in fact they are synthesized at equal levels in normal and castrated males. Specific endogenous lectins of mannose-6-phosphate and melibiose will be restored to their normal levels after injection of testosterone or dihydrotestosterone to castrated mice. Therefore, only the testosterone is capable of restoring the expression of specific receptors of xylose and mannose. As for dihydrotestosterone, it allows the recovery of synthesis of fucose specific endolectins.

Cytoplasm and percentage of labeled cells

Hormonal deprivation is associated with increased of expression of all cytoplasmic endolectins. Furthermore, only 20 to 80% of the epithelial cells expressed an increased binding. In castrated mice, heparin receptors are cytoplasmic and nuclear, half the cytoplasm of alveolar cells exhibited a strong binding with this probe, but in normal animals, or castrated treated by dihydrotestosterone, receptors are exclusively nuclear, and no cytoplasmic staining is detectable (Figure 1). After injection of the T or DHT, the labelling and the percentage of cells, expressing specific receptors for the mannose 6-phosphate, melibiose, mannose and xylose are identical to those observed in normal males. Specific cytoplasmic receptors of N-acetylglucosamine and N-acetylgalactosamine decreased after injection of one or the other exogenous hormone.

Basal membrane

Castration causes an increase in the synthesis of endolectins linking monosaccharides phosphorylated and sulfated glycosaminoglycans. The expression of these two endolectins reached the level of expression observed in normal males after injection of DHT to castrated mice. The three hormonal manipulations (hormonal deprivation, the injection of the T or DHT) have no effect on the expression of specific receptors of xylose and N-acetylglucosamine. Injection of the T allows an overexpression of endolectins linking mannose and α-L-fucose. The DHT activates the synthesis of specific endolectins of melibiose, N-acetylglucosamine and N-acetylgalactosamine.

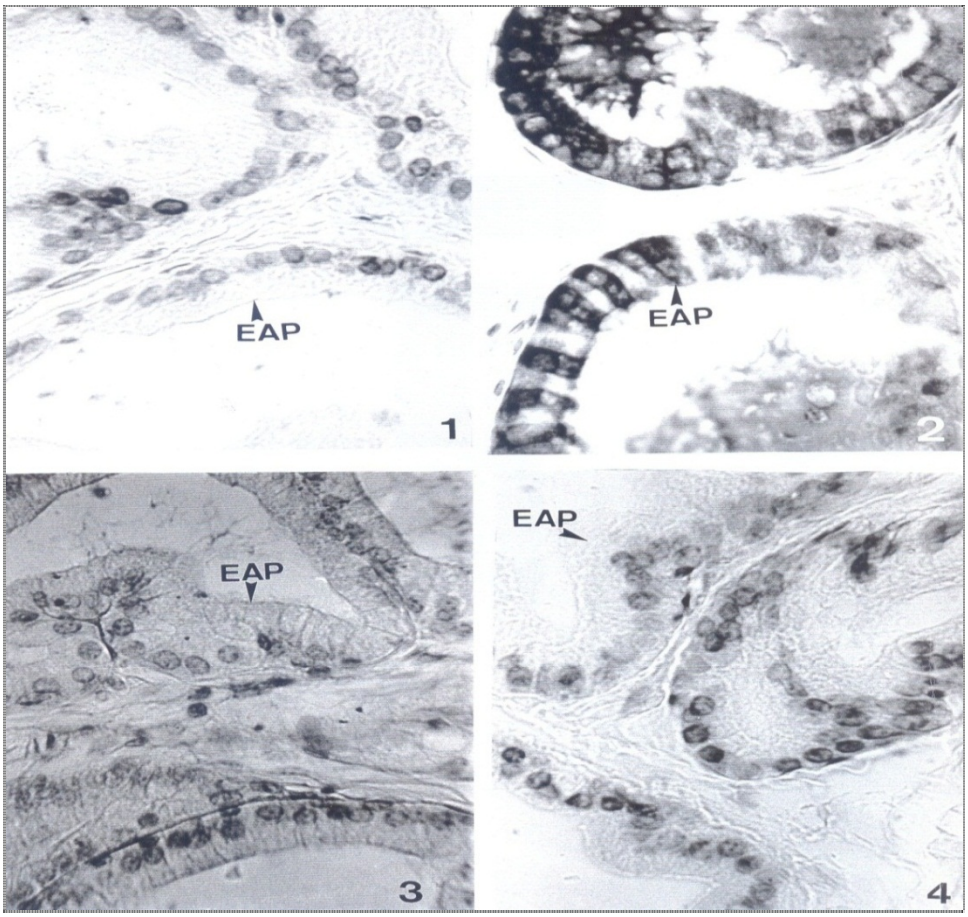

Figure 1. Binding sites of heparin in the ventral prostate of normal (1), castrated (2), castrated male injected with 5µg/g of testosterone (3) and castrated male injected with 1µg/g of dihydrotestosterone (4). EAP: Epithelium Alveolar Prostatic. No counterstaining x512.

Discussion

Neoglycoproteins has already proven it value for analysis on fixed tissue specimens (Coppée *et al.*, 1993; Danguy *et al.* 1998; Kayser *et al.*, 2003). To our knowledge, this report initiates the histochemical study of endogenous glycan binding proteins in adult male accessory sex organs. Our results clearly demonstrate specific and extensive binding of these synthetic probes to accessible sugar receptors, indicating the presence of carbohydrate binding proteins associated with alveolar glandular cells of the mouse prostate. In normal males endogenous receptors with specificity to xylose and mannose proved to be the most abundant at the luminal compartments of epithelial cells; in castrated males, there is an activation of all endolectines except those recognized by the mannose. T restores the expression of the endogenous receptors with specificity to xylose and melibiose, while DHT restores the expression of the endogenous receptors with specificity to heparin, fucose, melibiose and mannose-6-phosphate. At the apical epithelial cells, DHT seems more effective than T, regarding the synthesis of endolectines.In the cytoplasm, specific staining with neoglycoproteins was more faintly distributed, the castration activates all endolectines, the T doesn't activate them, but suppresses the receptors for the N-acetylated and,-fucosylated sugars, the DHT activates galactosyl receptors only.

Our reverse lectin histochemical method demonstrated a weak affinity of the basement membrane for all the synthetic probes used except for labelled heparin, whereas a moderate extent of receptor expression was visualized using xylose-BSA; in addition, no suppression of endolectines is observed following castration or injection of the T or DHT to castrated mice. Basement membranes are an essential contributor to epithelial

function and architecture. Together with stromal cells, basement membranes are essential for directing the formation of epithelial three dimensional acini and their functional differentiation (secretion of tissue specific proteins). The experiments presented here indicate that no significant differences in the expression of different endolectines at the basal membrane in the three experimental cases, which suggests that the synthesis of endolectines at this compartment is androgen independent.

Interestingly, a moderate concentration of receptors with specificity to sulphated polysaccharides (heparin) was observed only in the nucleus of normal mice glandular cells, the hormonal deprivation remove nuclear receptors of heparin, while the injection of T or DHT to castrated mice restores the expression of these endolectines. Such nuclear staining was also recorded in human placental tissues (Gabius & Bardosi, 1991) and fish epidermis (Danguy *et al*., 1991). Heparin and heparan sulphate play key roles in regulating many physiological processes (Ishihara, 1993). The heparin-binding activity may be involved in the translocation of basic fibroblast growth factor into the nucleus (Arese *et al*., 1999). The binding of labelled heparin, reported in nuclei in our study, may be a visual reflection of such an assumption. In this context, it is interesting to note that some internalized heparin sulphate is found in the nucleus (Yamaguchi, 1993).

Remarkably, after castration the binding of all the neoglycoproteins increases in a variable percentage (20%-80%) of glandular cells. The increased labelling was observed at both the luminal and intracellular compartments. Injection of the T or DHT makes the percentage of labeled cells to normal. Another interesting observation in the present study was that, during castration-induced regression, epithelia in the mouse prostate underwent an activation of expression of endolectines before castration. This observation indicates that, upon androgen depletion, prostatic epithelial cells are undergoing a series of metabolic changes which vary depending on the different parts of epithelial cells. Although no biochemical data on endolectins is available, it is in principe noteworthy that changes in the level of certain proteins have been documented in the rat. In detail, it has been shown that during its castration-induced regression the rat ventral prostate synthesized 33, 38 and 64 KDa proteins (Lee & Sensibar, 1987). Using an mRNA translation system, Montpetit *et al.* (1986) and Saltzman *et al.* (1987), observed the expression of unique proteins in the prostate of androgen-deprived rats.

From the results reported in this paper, it is conceivable that the various glycoconjugates, synthesized by the alveolar epithelial cells of the mouse prostate, are heterogeneous in their chemical composition. Furthermore, the hormonal status (hormonal deprivation, the injection of the T or DHT) changed the histochemical expression of endolectins.

Conclusion

The present work has revealed that neoglycoproteins have considerable potential for histochemically detecting the expression of endolectins, modulated by T and DHT. We were able to demonstrate not only the presence but also differences in the type and distribution of these specific binding sites in response to hormonal changes. So far the precise biochemical nature of endolectins synthesized by the prostate is unknown. Nonetheless, it is expected that glycohistochemistry will expand our understanding in this area and serve as a guideline for further work that attempts to determine the physiological significance of endolectins in the accessory sex glands.

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