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Effects of Testosterone and Dihydrotestosterone on glycans synthesis in mouse submandibular glands shown glycohistochemically

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Abstract

We studied histochemically the expression of glycans in the mouse submandibular glands of the normal, castrated, castrated mice injected with testosterone (T) and castrated mice injected with dihydrotestosterone (DHT). The avidin-biotin-peroxidase complex (ABC) technique was used on paraffin sections with a panel of biotinylated lectins. Our results show that (1) in all experimental cases, the acinar cells are s-WGA and PHA-L negative; in these structures, T or DHT repress the synthesis of the some glycan chains recognized by the GNA and PNA (2) in the excretory ducts (ED), the T restores the synthesis of only the Galβ(1-3)GalNAc recognized by the PNA, while the DHT restores the synthesis of this disaccharide and Neu5Acα(2,3)Gal recognized by MAA (3) In the granular convoluted tubule (GCT), no glycan chain is activated after injection of the T. The two hormones have antagonistic effects on the synthesis of Fucα(1-2)Gal and (α,β)GalNAc; indeed, T is as inhibitor and DHT as activator. We have attempted to correlate the glycohistochemical findings with recently published data obtained biochemically in the humans and rodents submandibular glands.

Keywords: Mouse submandibular gland, lectins, glycohistochemistry, testosterone, dihydrotestosterone.

Introduction

Glycosylation of the protein has a structural role and various functional in many specific biological functions, including the development of cancer (Hakomori & Cummings, 2012), viral and bacterial infections, autoimmunity, cell attachment to the matrix and the interactions extracellular protein-ligand in the cell (Burger-Calderona *et al.*, 2014). Glycosylation is the attachment of sugar moieties to the protein and it is a post-translational modification which is characterized by various glycosidic bonds, including N-, O- and C-linked glycosylation and phosphoglycosylation. Glycoproteins can be detected, purified and analyzed by different strategies, such as the use of lectins (Sharon & Lis, 1989). Lectins are proteins or glycoproteins from non-immune origins that agglutinate cells and/or precipitate complex carbohydrates, they are isolated from a wide variety of natural sources, both plant (Zuo *et al.*, 2012) and animal sources (Gold & Balding, 1975)). The affinity of different lectins to specific sugars makes them useful as histochemical probes (Akif *et al.*, 1993, 1994, 1995). Several lectin histochemistry studies of normal and pathologic conditions of salivary glands have been reported (Xu *et al.*, 2000; Nakadai *et al.*, 2012). The submandibular gland of the rodent is characterized by convoluted granular tubules, which are located between the intercalated and striated ducts. In the mouse, these convoluted granular tubules display sexual dimorphism; they are dominant in male mice, and smaller with a predominance of acini in female mice (Chretien, 1977). A recent study showed significant differences in gene expression profiles between men and women in human parotid tissue obtained from the histologically normal part of benign parotid tumors (Srivastava *et al.*, 2008). In addition, these glands are

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altered by various hormonal manipulations (Akif *et al.*, 1993). Surgical removal of the submandibular glands (sialectomy) in adult mice causes a profound drop in the plasma and tissue concentrations of nerve growth factor, and a reduction in the activity of tyrosine hydroxylase (Hendry & Iversen, 1973). In addition, the sialectomy leads to a decrease of luteinizing hormone (LH) and ultrastructural changes of interstitial Leydig cells (Boyer & Arancibia 1991). A relationship between the submandibular gland and thymus (Martinez *et al.*, 1973) on the one hand, and pineal gland (Boyer & Arancibia 1991) on the other hand could be demonstrated. However, a significant component of salivary gland secretions is found in blood indicating its endocrine role and its role in maintaining the homeostasis (Mathison, 1995; Mathison, 2009). Furthermore, sialectomy at mice allows the removal of a breast adenocarcinoma A10 (Barry *et al.,* 1975). Sialolithiasis is composed of varying ratios of organic and inorganic substances; the organic substances are glycoproteins and mucosaccharides (Ashby, 1995). It mainly develops in the ductal system of the submandibular gland, which has got highest predilection for sialolithiasis with 80% occurrence rate, followed-by 19% in the parotid and 1% in the sublingual glands (Batori *et al.*, 2005); males are affected twice as much as females (Nahlieli *et al.*, 2000; Siddiqui, 2002); this is explained by the predominance of tubular structures in males and acinar ones in females.

The objective of the present study, which is the first of its kind, is to focus on the glycohistochemical aspect of mouse submandibular glands in normal as well as of steroid deprived mice, and to compare it with the effects of administration of exogenous hormones such as T or DHT; for this purpose we used a panel of biotinylated lectins on Bouin's fixed, paraffin-embedded tissue sections according to the glycohistochemical technique developed by our research team (Akif *et al.*, 1993, 1994, 1995).

Materials and Methods

Animals and preparation of tissues

Sexually mature NMRI mice (8 weeks old) weighing 20-25 g, were castrated under sodium pentobarbital anesthesia, and used for experiments eight weeks later. The castrated mice were injected with 5µg/g of testosterone or with 1µg/g of dihydrotestosterone for 5 days. 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 anesthetized with ether. The submandibular glands were excised and promptly cut transversely through the mid-region into small blocks. They were exposed and bathed with the fixative during dissection. Submandibular glands were fixed in Bouin's solution for 24h and embedded in paraffin and sectioned at the thickness of 5µm. After dewaxing, lectin histochemical staining was performed using the avidin-biotin method, following optimized procedures (Akif *et al.*, 1993, 1994, 1995).

Lectin histochemistry

Nine types of biotinylated lectins, purchased from Vector Laboratories (Burlingame, CA, USA) were used. Their full names, abbreviation, natural sources, saccharide specificities and binding inhibitors are listed in table 1. After deparaffination, tissue sections were incubated in methanol/0.3% H_2O_2 for 30 min at room temperature to block endogenous peroxidase activity. Sections were then washed in PBS (phosphate-buffered saline, 0.15M NaCl containing 0.01M phosphate buffer, pH 7.3±0.1) and incubated with biotinylated lectins (5µg/ml) for 10 min at room temperature. Then the sections were rinsed in PBS and incubated for 30 min with an avidin-biotin-

peroxidase complex (ABC kit, Vector). After being washed with PBS, the sections were developed in 3,3'-diaminobenzidine 4HCl (DAB, Sigma)-H_2O_2 medium under microscopic control at room temperature to visualize the activity of peroxidase. The sections were rinsed with tap water, counterstained with haematoxylin, dehydrated, cleared and mounted with DPX. Controls for lectin binding included: 1- omission of the respective lectin; 2- omission of the ABC kit reagents; 3- incubation of the sections with lectin solutions to which 0.2-0.3M of the specific sugar (Janssen Chemica, Beerse, Belgium) (Table 1) had previously been added. In order to block nonspecific binding of biotin-avidin system reagents, sections were incubated with a blocking kit (vector lab) just prior to the addition of lectin conjugates. The localization of lectin-specific carbohydrates in the tissues by the probes was examined independently by two investigators who subjectively rated the intensity of binding as negative (0) to very strong (3) (Table 2).

Table 1. Lectins used for histochemical characterization of glycans.

Latin name	Lectin Symbol	Sugar residues or sequences recognized by lectins	Sugars found to inhibit histochemical binding
Galanthus nivalis	GNA	Manα(1-3)Man	Man
Ulex europaeus	UEA-I	Fucα(1-2)Gal	L-Fuc
Sambucus nigra	SNA	Neu5Acα(2-6)Gal	Neu5Ac
Maackia amurensis	MAA	Neu5Acα(2,3)Gal=Neu5Acα(2,3)GalNac	Neu5Ac
Arachis hypogaea	PNA	Galβ(1-3)GalNAc	Lactose
Ricinus communis	RCA-I	Galβ(1-4)GalNAc	Gal
Glycine maximus	SBA	Terminal (α,β)GalNAc	GalNAc
Succinylated *Triticum vulgare*	s-WGA	GlcNAc[β(1-4)GlcNAc]1-2	GlcNAC
Phaseolus vulgaris	PHA-L	GlcNAcβ(1-2)Man	Complex

GlcNAc: N-acetylglucosamine, GalNAc: N-acetylglactosamine, Gal: galactose, Man: mannose, Glc: glucose, L-Fuc: fucose, Neu5Ac: neuraminic acid.

Results

The binding patterns observed with each lectin are summarized in table 2.

Acinar cells (A)

The castration suppresses the synthesis of Fucα(1-2)Gal, Galβ(1-3)GalNAc and (α,β) GalNAc recognized by the UEA-I, PNA (Fig. 1) and SBA, respectively; Furthermore, hormonal deprivation had no effect on the synthesis of the glycan chains recognized by SNA, MAA, s-WGA and PHA-L. The injection of the T to castrated mice does not restore the expression of any glycan chain; while the DHT restores the synthesis of only (α, β)GalNAc recognized by the SBA.

Excretory (striated) ducts (ED)

After castration there is an inhibition of the expression of Neu5Acα (2,3)Gal, and activation of Manα(1-3)Man and Galβ(1-3)GalNAc; the labeling with UEA-I, SNA, RCA, SBA, s-WGA and PHA-L remains unchanged. T does not restore the synthesis of the disaccharide Galβ(1-3)GalNAc recognized by the PNA (Fig. 1);while DHT restores the synthesis of this disaccharide and Neu5Acα(2,3)Gal recognized by the MAA.

Granular convoluted tubules (GCT)

Lectin histochemistry revealed a broad spectrum of carbohydrate moieties within the cells of the GCT. Only the labelling with GNA and s-WGA increased after castration, and the labelling with PHA-L decreased. Synthesis of Fucα(1-2)Gal and (α,β)GalNAc decreases after injection of T, and increases after injection of DHT to castrated mice.

Table 2. Semi-quantitatively-determined intensity of lectin binding to the mouse submandibular gland.

Type of lectins	Normal males			Castrated males			Castrated males +5µg/g testosterone			Castrated males + 1µg/g dihydrotestosterone		
	GCT	ED	A	GCT	ED	A	GCT	ED	A	GCT	ED	A
GNA	2	1	3	3	2	3	3	2	1	3	2	1
UEA-I	2	2	2	2	2	0	1	2	1	1	2	1
SNA	0	0	0	0	0	0	0	1	1	0	1	1
MAA	0	1	1	0	0	1	0	0	2	0	1	0
PNA	3	1	3	3	2	0	3	1	1	3	1	1
RCA-I	2	1	3	2	1	1	2	2	1	2	2	1
SBA	2	1	3	2	1	0	1	1	2	1	1	3
s-WGA	0	2	0	2	2	0	2	2	0	2	1	0
PHA-L	2	1	0	1	1	0	1	1	0	2	1	0

GCT: granular convoluted tubule; ED: excretory duct; A: acinus. Numbers indicate intensity on an estimated scale from 0 (unreactive) to 3 (strongly reative).

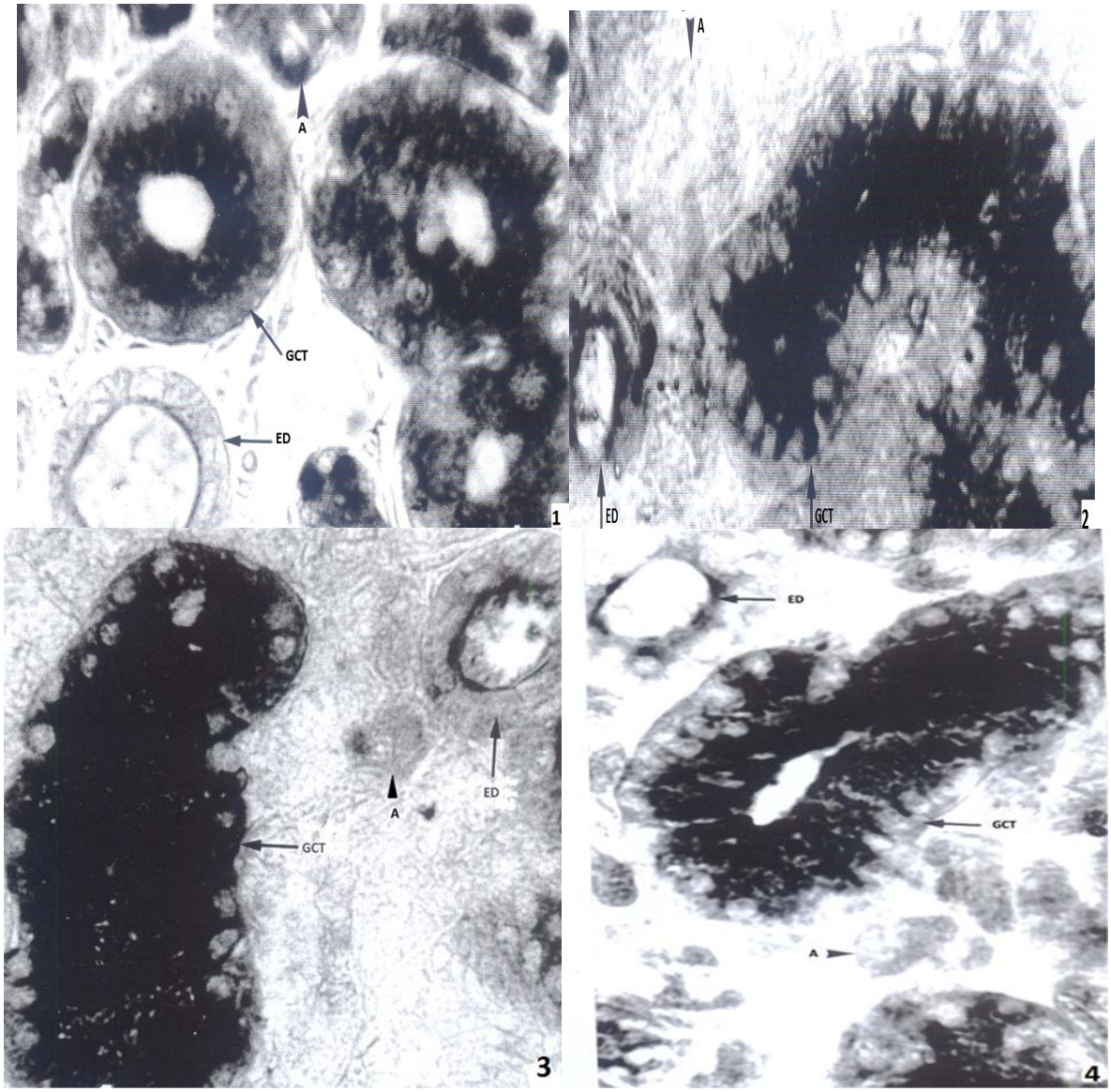

Figure 1. Binding sites of the PNA in the submandibular gland of normal (1), castrated (2), castrated male injected with 5µg/g of testosterone (3), and castrated male injected with 1µg/g of dihydrotestosterone (4). ED: excretory ducts, GCT: granular convoluted tubules, A: acini. No counterstaining. X 510.

Discussion

In normal males, the acinar cells of submandibular glands do not express the Neu5Acα(2-6)Gal disaccharide, but synthesize Neu5Acα(2,3)Gal at low concentrations, this shows the high specificity of the two used lectins namely *Sambucus nigra* (SNA) and *Maackia amurensis* (MAA); some results suggest that the incorporation of neuraminic acid in glycoprotein side chains are more advanced in central than semilunar acinar cells of the ferret submandibular gland (Triantafyllou *et al.*, 2004).The castration has no effect on the synthesis of neuraminic acid recognized by SNA and MAA, and the injection of the T or DHT to castrated mice activates the synthesis of Neu5Acα(2-6)Gal recognized by SNA. Furthermore, the DHT inhibits expression of Neu5Acα(2,3)Gal. This shows that the androgens, at least some of them, are necessary for the maintenance of expression of the two neuraminic chains in acinar cells. Using another lectin specific of neuraminic acid (*Limax flavus agglutinin* = LFA), Schulte (Schulte, 1987) visualized strong labeling on acinar cells, arguing for the presence of a high concentration of sialylated glycoproteins. This discrepancy is probably due to a genetic difference of used animals. Also other studies realized on the Swiss strain mice show a background or a weak labeling with the same lectin in the acini (Menghi *et al.*, 1991).

Cellular glycoconjugates are known to be modified with the development, differentiation and maturation of cells (Kim & Allen, 1994; Ito *et al.*, 1995; Accili *et al.*, 1999).The present study has elucidated that the glycoconjugates synthesis changes in each cell structure of the salivary gland of mice in response to hormonal manipulations. Numerous physiological studies have concluded that salivary acinar cells release the primary components of saliva (Young *et al.*, 1987). In all experimental cases the acini are s-WGA and PHA-L negative, which proves the total absence of N-linked oligosaccharides containing GlcNAcβ(1-4)GlcNAc and GlcNAcβ(1-2)Man at these structures, and a hyperactivity of β-N-acetyl-D-glucosaminidase at the acinar cells. A biochemical study of the submandibular gland of the rat (Przybylo *et al.*, 2004) showed that this enzyme decreased according to increasing age of the animal, this decrease occurs mainly in GCT and ED cells; and not in the acinar ones, since we were able to visualize a labeling with s-WGA and PHA-L in ED and a labeling with PHA-L in GCT; our glycohistochemical study is more accurate than biochemical one, because the histochemistry allows studying each structure separately. Furthermore it has been shown that sequences recognized by PHA-L, reflect dysfunction in the glycan synthesis, culminating in the formation of terminal oside structures that favor the metastatic spread of tumor cells (Guillot *et al.*, 2004).

In the excretory ducts, and in all experimental cases there was a moderate labeling with UEA-I, this suggests that the routing of Fucα(1-2)Gal disaccharide to the target organs is not affected by the different hormonal manipulations. Using the UEA-I, other authors have highlighted low quantities of L-fucose in the excretory ducts of the rat submandibular gland (Zhang *et al.*, 1994); of man (Born *et al.*, 1987), but no traces of L-fucose in mice (Menghi et *al.*, 1991), these latter state that the UEA-I has no affinity for the mouse submandibular gland (Swiss strain), while these authors use the UEA-I at high concentration (20 µg/ml) instead of 5µg/ml used in our case; this disagreement may have come from the fact that these authors set their samples in Cornoy which has a pernicious effect on the glycan chains of the submandibular glands (Takai *et al.*, 1986).

The granular convoluted tubules (GCT) do not react with SNA and MAA, this underlines the total lack of Neu5Acα(2,3)Gal and Neu5Acα(2-6)Gal

expression; this latter disaccharide is considered as a marker of cancer tissues (Springer, 1984) and as the recognition site of H3N2 influenza virus, the interaction virus-Neu5Acα(2-6)Gal is facilitated by the methyl groups of the neuraminic acid (Lamblin *et al.*, 1991). Given the importance of granular convoluted tubules in the maintenance of submandibular gland physiology, it seems appropriate that they do not express these receptor types to escape any viral attack.

Castration or injection of T or DHT to castrated mice, active the addition of Manα(1-3)Man at excretory ducts and granular convoluted tubules; the finding which indicates the presence of mannose in the saliva (Berger *et al.*, 1982; Reddy *et al.*, 1982, support our observation regarding the presence of mannose in the mucous and serous cell types whatever the hormonal status. At the acini, in normal males the Manα(1-3)Man expression is maximum; castration has no effect on the expression of this disaccharide; while the T or DHT inhibits it. The mannose in the serous and mucous acinar cells has also been shown to be major constituents of the secreted mucous glycoproteins (Tabak *et al.*, 1982). These glycoproteins bind to the tooth surface forming part of the acquired enamel pellicle and have been shown to agglutinate specific strains of oral microorganisms (Hogg & Embery, 1979; Kornfeld & Kornfeld, 1985).

If we consider all the submandibular gland, it is noted that the injection of exogenous hormones such as T (5µg/g) or DHT (1µg/g) to the castrated mice have the same effect on the synthesis of Manα(1-3)Man, Fucα(1-2)Gal, Neu5Acα(2-6)Gal,Galβ(1-3)GalNAc and Galβ(1-4)GalNAc recognized by GNA, UEA-I, SNA, PNA and RCA-I, respectively. The similarity of expression of these glycoconjugates after injection of the T or DHT is explained by the fact that the submandibular glands contain the same receptor levels for these two hormones (Yorichika, 1989).

Conclusion

Glycohistochemical analysis of the mouse submandibular glands revealed that the injection of T or DHT have the same effect on the synthesis of Manα(1-3)Man, Fucα(1-2)Gal, Neu5Acα(2-6)Gal, Galβ(1-3)GalNAc and Galβ(1-4)GalNAc. In all test cases, the acini were succinylated WGA and PHA-L negative; the staining with SBA and PHA-L in the excretory ducts remains constant, in addition, the expression of glycoconjugates recognized by the SNA, MAA, PNA and RCA-I in granular convoluted tubules remains invariable.

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