·*Original Article*·

Localization and potential function of androgen receptor in rat salivary gland

Hong-Jun Li[1], Bing Yao[1], Wei Liang[2], Yong-Mei Wang[4], Qi-Lu Xu[3], Yu-Feng Huang[1]

[1]*Department of Reproduction, Nanjing Jinling Hospital, Clinical School of Medical College, Nanjing University, Nanjing 210002, China*
[2]*Department of Chinese Traditional Medicine of No. 454 Hospital, Nanjing 210002, China*
[3]*Nan-Kong Hospital, Nanjing 210002, China*
[4]*Jiangsu Family Planning Research Institute, Nanjing 210008, China*

Abstract

Aim: To investigate the localization and quantity of androgen receptor (AR) in the salivary glands of rats with further analysis on the effect of castration. **Methods:** Sixty male Wistar rats, aged 30–60 days, were randomly divided into three groups (castrated, sham-operated and normal controls) with 20 rats in each group. The rats in the castrated group were castrated and the submaxillary glands were removed after 1 week. The salivary glands of the rats in the sham-operated and the normal control groups were also removed. Parts of the salivary glands were fixed for immunohistochemistry and *in situ* hybridization assays. Other parts were used for Western blot. **Results:** AR immunoreactivity in the three groups was localized in the glandular epithelial cells of the serous acinus and the glandular duct of the salivary gland, mainly in the nuclei. AR mRNA hybridization signals in the salivary glands of the castrated group were mainly distributed in the epithelial cells of the convoluted and secretary ducts; AR mRNA in the sham-operated and the normal control groups were found in the epithelial cells of the convoluted, the secretary and the excretory ducts. The quantity of AR in the salivary glands was decreased significantly in the castrated rats compared with the sham-operated and the normal controls. Moreover, epidermal growth factor (EGF) secreted by the salivary glands was also decreased in the castrated rats. **Conclusion:** Castration appears to affect the production of AR in the salivary gland and the distribution of the AR mRNA and could further affect the function of the salivary gland. The changes of AR and the distribution of AR mRNA may play an important role in the interactions between the testes and the salivary gland. *(Asian J Androl 2005 Sep; 7: 295–301)*

1 Introduction

The androgen receptor (AR), a member of the steroid/nuclear receptors superfamily, is a ligand-dependent transcription factor that increases or decreases the expression of its target genes and is involved in the regulation of development, differentiation and maintenance of

Correspondence to: Dr Bing Yao, Department of Reproduction, Nanjing Jinling Hospital, Clinical School of Medical College, Nanjing University, 305 East Zhongshan Road, Nanjing 210002, China.
Tel: +86-25-8086-0174, Fax: +86-25-8481-5730
E-mail: yaobingliang@yahoo.com.cn

male reproductive functions as well as in the generation of sexually dimorphic characteristics in nongenital tissues. Upon androgen binding, the AR acquires a new conformational state that renders it to be capable of interacting with the specific steroid response element. Liganded AR is also able to interact with other transcriptional factors and coactivators. The coactivators further recruit complexes with chromatin remodeling activities [1]. AR is reported to colocalize with epidermal growth factor receptor (EGF-R) at the membrane of the prostate cancer cell line, LNCaP. Androgen has an effect on epidermal growth factor (EGF)-mediated signaling related to invasion in LNCaP [2]. Some genes are involved in the effect of androgen on the modulating secretion of EGF in this cell line, such as prostin-1, urokinasetype plasminogen activator and neutral endopeptidase [2]. Recently, AR has been shown to be associated with c-Src kinase family membranes and caveolin-1 in LNCaP. Both src and caveolin-1 are also capable of interacting with EGFR and are involved in EGF-stimulated signaling [3].

Studies found that the mature dysfunction of the sperm, ultrastructural changes of the Leydig cells and a decrease in plasma luteinizing hormone (LH) level happened after the salivary gland of the rat was removed [4]. These changes can be reversed by administration of EGF that is produced mainly in the salivary gland [5]. Furthermore, testosterone could increase the EGF expression [6] and change the amylase activity in the salivary gland. The EGF mRNA levels could fall after male mice were castrated [7]. So it is hypothesized that an axis might exist between the testis and the salivary gland [8].

Our previous studies demonstrated that gonadotropin-releasing hormone (GnRH) could stimulate the secretion of EGF from the salivary gland [9]. GnRH receptor and AR were co-localized in the salivary gland [10]. AR mRNA was distributed in the glandular epithelial cells of the serous acinus and the epithelial cells in all gland ducts [11]. These data suggested that: 1) AR could be produced by the salivary gland; 2) in the salivary gland, AR is localized in the epithelium from which the EGF is secreted; 3) androgen might bind to AR to modulate the secretion of EGF as well as GnRH. In addition, because androgen often regulates the expression of its own receptor [12], castration may also be expected to affect the AR in the salivary gland. The present experiment employed immunohistochemical and *in situ* hybridization methods to investigate the localization of AR and the

distribution of AR mRNA in the salivary gland following castration and on the level of EGF in the salivary gland.

2 Materials and methods

2.1 Reagents

Nitro-blue-tetrazolium (NBT) and 5-bromo-4-chloro-3-indilyl-phosphate (BCIP) were supplied by the Department of Histology and Embryology of the Second Military Medical University. The ABC kit was a Vector product (Burligame, CA, USA). The *in situ* hybridization kit was purchased from the Boster Company in Wuhan, China. The Digoxigenin-labeled AR oligonucleotide probe was produced from TaKaRa Biotechnology (Dalian) Co., Ltd. (Dalian, China). The sequence of the probe was, 5'-ctggttgtca ctacggagct ctcacttgtg cagctgcaa-3', which was used for AR mRNA *in situ* hybridization. The polyclonal AR antiserum (PG-21) was from UpState Biotech (Lake Placid, NY, USA). Antiserum produced against EGF(3124) in rabbit was purchased from Wakunaga Co. (Hiroshima, Japan). The anti-rabbit IgG-peroxidase and BM blue POD substrate were from Roche (Indianapolis, IN, USA). The densitometric analysis was performed with Image Gauge software (Fuji, Tokyo, Japan).

2.2 Animals and sample collection

Sixty male Sprague–Dawley rats (200 g–250 g, postnatal 30–60 days) bred in our hospital were used for the present experiments. Rats were maintained in light (5:00–19:00) and temperature (23 ℃ ± 3 ℃) controlled rooms and allowed free access to food and tap water. All procedures employed in the present study were done following the guidelines for animal treatment of Nanjing Jinling Hospital, which is in accordance with the principles and procedure of the NIH guide for the care and use of laboratory animals. The animals were randomized into three groups of 20 rats each. One group was used as unoperated controls, another group was sham-operated, and the third group was surgically castrated under anesthesia as previously described [13]. After one week, all rats were sacrificed at the same time of the day by decapitation. The salivary gland was quickly removed and divided into two parts. One part was immediately fixed in Bouin's solution for 12 h, embedded in paraffin, then 5-μm sections were prepared. The other part (approximately 300 mg) was suspended in 1 mL 0.01 mol/L PBS, pH 7.4, and homogenized (3 × 10 s bursts on ice) with a Polytron homogeniser. Then this suspension was

centrifuged at $10000 \times g$ for 20 min at 4 ℃. The supernatant was taken and quantified. The protein was then kept at –20 ℃.

2.3 AR immunohistochemical procedure

The paraffin sections were dewaxed, hydrated and incubated in methanol-H_2O_2 for 20 min to remove endogenous peroxidase. They were then stained according to the immunohistochemical ABC method. Tissue sections were incubated at 4 ℃ for 24 h in the primary antibodies of PG-21 (1:1000 dilution). The secondary antibody, biotin-labeled goat anti-rabbit IgG (1:200 dilution), was incubated at room temperature for 1 h, and ABC complex (1:100 dilution) was incubated at room temperature for 30 min. The negative control tissue sections were subjected to the same procedures except that the diluted rabbit serum replaced the first antibody.

2.4 Hybridization procedure

The *in situ* hybridization of AR mRNA was carried out on the paraffin sections. The sections were dewaxed, hydrated, and then rinsed in distilled water three times for 5 min each time, and in proteinase K (1 μg/mL) at 37 ℃ for 20 min. The sections were post-fixed in 4 % paraformaldehyde for 5 min, and rinsed in distilled water three times for 5 min each time. The hybridization buffer containing the AR oligonucleotide probe (2.0 μg/mL) was applied to the appropriate sections and hybridization was conducted in a sealed humid box at 43 ℃ for 12–18 h. The sections were washed in 2 × saline sodium citrate (SSC) at 37 ℃ twice for 5 min each time, then in 0.1 × SSC at 37 ℃ for 10 min, incubated in mouse anti-Dig antibody at 37 ℃ for 2 h, rinsed in 0.5 mol/L TBS three times for 2 min each time, incubated in biotin labeled goat anti-mouse IgG at 37 ℃ for 1 h, rinsed in 0.5 mol/L TBS three times for 2 min each time, incubated in SABC-AP at 37 ℃ for 30 min, and rinsed in 0.5 mol/L TBS four times for 5 min each time. The alkaline phosphatase reaction was conducted by incubation with the complex solution of NBT and BCIP for 30–50 min. The sections were washed in water to stop the reaction, then dehydrated in alcohol, cleared in xylene, and mounted on Canada balsam.

The negative controls were processed as described above but in the absence of the labeled probe and were incubated with normal mouse serum instead of mouse anti-Dig antibody. An additional control group of *in situ* hybridization was carried out as previously described in which the sections were treated with labeled probe mixture in the presence of a 100-fold excess concentration of unlabeled probe mixture [14].

2.5 Western blot

Following the above procedure, 30 μg of each sample was separated with 12 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel, and then transferred onto immun-blot™ PVDF membrane (Hercules, CA, USA). The membrane was then blocked and incubated with PG-21 (1:1000 diluted with blocking solution) or anti-EGF (3124) antibody (1:1000 diluted with blocking solution) at room temperature for 1 h. After three 5-min washes, the membrane was incubated with anti rabbit IgG-Peroxidase antibody (1:1000 diluted with blocking solution) at room temperature for 1 h. After a further three 10-min washes, the BM blue POD substrate (Indianapolis, IN, USA) was added and the blue band appeared. A picture was taken and Image Gauge software was used to analyze the density of each band.

2.6 Statistical analysis

The results were evaluated statistically using Student's t-test and $P < 0.05$ was considered significant.

3   Results

3.1 Localization of AR in salivary gland

AR is dark brown in color with a light yellow, non-staining background in AR immunoreactive positive cells (Figure 1). The glandular epithelial cells of the serous acinus and the epithelial cells of the secretory duct, the excretory duct and the granular convoluted tubule showed AR immunoreactivity and the immunoreactive materials were mainly distributed in the nuclei. The distribution of AR in salivary glands is similar in castrated, sham-operated and normal groups (Figure 1).

3.2 Distribution of AR mRNA in rat salivary gland

The AR hybridization signal of dark blue color can be easily identified (Figure 2). AR mRNA hybridization signals were detected in the epithelium of the salivary gland serous acinus, but not in the mucous acinus. Epithelial cells in the intercalated duct and the secretory duct in castrated rats exhibited AR mRNA hybridization signals, which were present in cytoplasm but not in nuclei, but the signals of AR mRNA in the sham-operated and the normal control groups were distributed in the epithelial
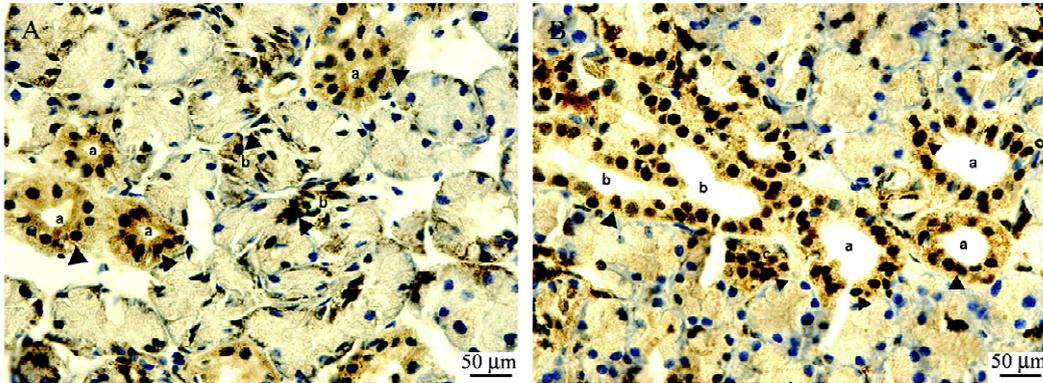
Figure 1. Immunohistochemical localization of androgen receptor in a rat salivary gland after castration is similar to that in the sham and control groups. (A): Sections of rat salivary gland after castration, reacted with PG-21 and counterstained with hematoxylin. The epithelium of serous acinus (a), and epithelial cells of the secretory duct (b), showed androgen receptor positive immunoreaction. (B): The epithelial cells of the secretory duct (a), the epithelial cells of the excretory duct (b), and the serous acinus (c) showed androgen receptor positive reaction. The positive substances were mainly distributed in the nuclei (▲) × 400.
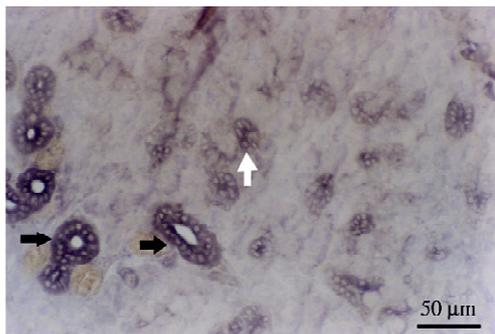

Figure 2. Androgen receptor mRNA in a rat salivary gland after castration was analyzed by *in situ* hybridization. Androgen receptor mRNA is distributed in the secretory duct (⬆) and the epithelial of the convoluted duct (⇧), the positive substance was distributed in cytoplasm with the negative nuclei × 400.

cells of intercalated duct, secretory duct and excretory duct. The patterns were the same as that we have reported previously [11] (data not shown).

3.3 Effects of castration on the expression of AR and EGF in salivary gland

The expression of AR and EGF in the salivary gland was analyzed by Western blot. As shown in Figure 3, castration resulted in a significant decrease in AR compared with that in the sham operation and the normal control groups ($P < 0.05$). EGF was also decreased significantly in the castrated rats compared with those in the sham-operated and the normal control rats ($P < 0.05$) (Figure 4).

4   Discussion

Salivary proteins come in a variety of different forms with diverse functions. In some cases, a salivary protein's function has yielded to biochemical studies. For example, salivary amylase digests dietary starches, glycoproteins lubricate hard and soft tissues, and some peptides retard the growth of bacteria and fungi in the oral cavity. For other salivary proteins, however, the function has remained elusive. AR and androgen binding protein (ABP) are the two of those. Verhoeven and Wilson [15] found an androgen-binding complex to exist from birth and throughout the life of normal mice that could be precipitated at 40 % saturation with ammonium sulfate. Studies demonstrated that ABP is produced *de novo* in the submaxillary gland and to be composed of two different disulfide-bridge subunits, alpha (*ca*. 12 000 daltons) and beta (*ca*. 8800 daltons). ABP binds with the male sex steroid hormone in mouse salivary glands [16], but does not precipitate out of saliva before 70 % ammonium sulfate saturation, indicating that the AR and mouse salivary ABP are not the same protein.

Sawada and Noumura [17] using anti-AR antibodies found that the specific immunoreactivity to AR appeared only in the cell nuclei of the acini, the intercalated ducts,
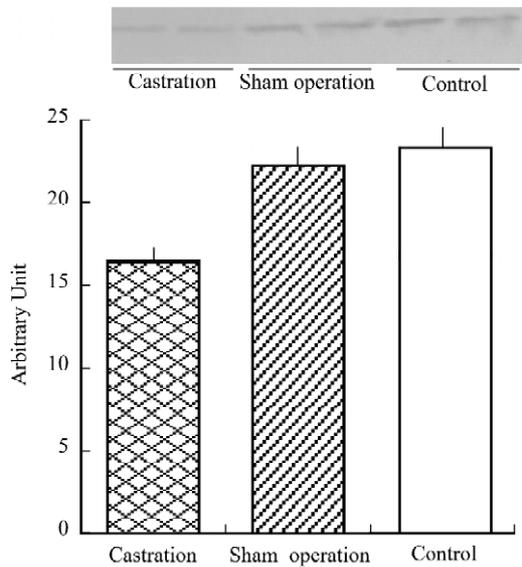
Figure 3. The western blot analysis of AR in the salivary gland was performed as described in the materials and methods section. Results are expressed as in arbitrary units and the bars represent the mean ± SD of 20 independent experiments, each performed in triplicate. This graph only shows the results from two independent experiments from three groups respectively. Student's *t*-test indicated significant differences comparing the castrated groups with the sham-operated group and the control group, *P* < 0.05.
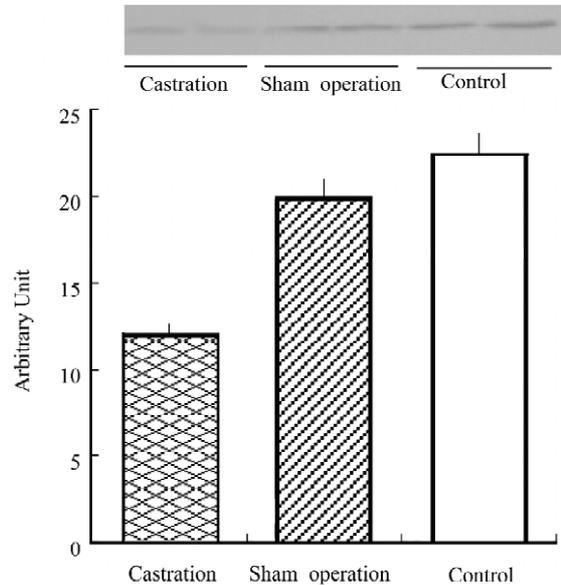

Figure 4. The western blot analysis of EGF in the salivary gland was performed as described in the material and methods section. Results are expressed as arbitrary units and the bars represent the mean ± SD of 20 independent experiments, each performed in triplicate. This graph only shows the results from two independent experiments from three groups respectively. Student's *t*-test indicated significant differences comparing the castrated groups with the sham-operated group and the control group, *P* < 0.05.

the granular convoluted tubules (GCT) and the excretory striated ducts in the salivary glands of mice of both sexes. This result is consistent with our previous findings in the salivary gland of the male rat using PG-21 [10]. This distribution suggested that AR might have the biologic function in salivary gland.

It is reported that androgen often regulates the expression of its own receptors [12], indicating a biologic relationship exists, but, the effect of castration on AR in extra-testis organs such as the prostate or salivary gland remains obscure. Kumar *et al*. [18] reported that the levels of AR mRNA in the prostate increased following castration. The maximum level was achieved in 5 days, and a decline in the level of AR mRNA was observed after 5 days. However, Husmann *et al*. [19] found AR in the prostate decreased 24 h after castration. Nagai *et al*. [20] found AR mRNA increased in the castrated male mice, while the administration of testosterone reduced the AR mRNA in female mice, and suggested that the amount of AR mRNA in mouse salivary gland can be reduced by androgen.

In our present study, we used 1-week-old castrated rats and found AR immunoreactivity decreased. The localization of AR after castration is similar to that in our two controls. This result is consistent with Sawada and Noumura's [17] findings that 1-week castration did not cause any changes in the intracellular distribution of androgen receptor and the percentage of immunoreactive cells in each region of the adult salivary gland. The reasons our results are different from other reports [21] might be: 1) the method for tissue preparing is different (we utilized Bouin's solution, which can protect the protein from being lost from the tissue); and 2) the antibody is different (PG-21 is a polyclonal antibody raised against a synthetic peptide corresponding to the first 21 N-terminal amino acids of the androgen receptor). The exact reason should be further clarified by comparing the distribution of AR in different tissues using this fixed method and PG-21 to those of Sar *et al*. [21]. This work is currently being done in our laboratory.

The hybridization signals of AR mRNA were detected in glandular epithelial cells of serous acinus and epithelial cells but not in epithelial cells of the excretory duct in the castrated rats. This different distribution compared to AR immunoreactivity may reflect a modulation of the transcript of AR mRNA in the salivary gland. We, hence, suggest that the decrease of AR in castrated rats may be caused by the change in distribution of AR mRNA. Our laboratory is planning to investigate the detailed mechanism by organ culture and to further study the quantity of AR mRNA using real-time reverse transcriptase PCR (RT-PCR) to clarify the mechanism of the decrease of AR after castration.

EGF is a potent regulator of cell growth and differentiation in many tissues, and its level is regulated by several hormones in a tissue-specific fashion. Testosterone can increase EGF levels in the salivary glands. Treating female mice with testosterone first raises the level of prepro-EGF messenger RNA in the salivary gland 3 days after the treatment, and the levels approach those in the male after approximately 1–2 weeks [6]. The castration of adult male mice can cause EGF mRNA levels in the salivary gland to begin to fall within 24 h [6]. In the present study, we found a decrease of EGF in the salivary gland 1 week after castration. Because this decrease is accompanied by the decrease of AR, we hypothesized that the decrease in the expression of EGF in the salivary gland might be related to that of AR.

More and more evidence indicates that the salivary gland exerts not only an exocrine function but also an endocrine function. For the exocrine function, the salivary gland secretes many enzymes in the oral cavity to exert many digestive functions; for the endocrine function, it can synthesize EGF and nerve growth factor (NGF). Recent studies revealed that the salivary gland is closely related to the testis. EGF secreted mainly from the salivary can modulate the spermatogenesis as an autocrine and/or paracrine factor. Aida *et al.* [22] reported that EGF played a role in male germ cell development and suggested that EGF might be involved in the paracrine control of spermatogenesis *in vivo*. Removing the salivary gland can also cause a decrease in testosterone secreted from the testis [23]. On the other hand, testosterone can increase the expression of EGF from the cultured salivary gland [6]. The secretion of amylase from the salivary gland and the production of EGF mRNA in salivary glands were decreased by castration [7]. If the functional relationship between the

salivary gland and the testis can be clarified, it will help us to understand the detailed function of the testis and to further modulate the function of the testis involved in reproduction. The detailed mechanism of the modulation in androgen, AR and the EGF mRNA in the salivary gland after castration needs to be further studied.

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