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ORIGINAL PAPER

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Gubernacular fibroblasts express the androgen receptor during testis descent in cryptorchid rats treated with human chorionic gonadotrophin

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Abstract Cryptorchidism was provoked in 3 day old rats treated with 17-β-estradiol over 30 days to identify the cells that express the androgen receptor (AR) during experimental testis descent in the gubernaculum. In one group of animals, testis descent was induced with human chorionic gonadotrophin (hCG) applied daily for 5 or 10 days. A correlative study using a testosterone radioimmunoassay with electron microscopy and immunocytochemical detection of AR was performed in gubernacula of hCG treated and untreated control animals. The gubernaculum of rats undergoing testes descent showed a dramatic increase in the number of AR-positive cells. These were located in the connective tissue among smooth muscle cells in the gubernacular cord and between striated muscle fibers in the bulb. In both regions, the AR-positive cells were identified as fibroblasts. Several clusters of amorphous material appeared in the extracellular matrix of the connective tissue in hCG treated rats. Our results suggest that testosterone induces the expression of AR in gubernacular fibroblasts which seem to degrade the extracellular matrix during gubernacular involution.

Keywords Cryptorchid · Gubernaculum · Androgen receptor · Testis descent · Fibroblasts · Human chorionic gonadotrophin

Introduction

The failure of testes to descend into the scrotum, known as cryptorchidism, affects approximately 3% of male children [1]. During development, testicular descent occurs in two phases: transabdominal and inguinoscrotal [2]. In the rat, the transabdominal phase occurs during days 16–19 of gestation and the inguinoscrotal phase starts at day 19 of gestation; complete descent is accomplished around day 21 after birth [3, 4].

The gubernaculum, first described by John Hunter [5] as a fibrous structure that anchors the testicle and the epididymis to the floor of the scrotum, is required for testis descent. In the rat, the gubernaculum forms between days 14 and 15 of gestation [3]. In this species, the gubernaculum has two regions: the cranial gubernacular cord connecting the testicle and the epididymis and the caudal gubernacular bulb expanding to the retroabdominal wall. Histologically, the gubernaculum is composed of mesenchymal cells and abundant extracellular matrix, rich in glycosaminoglycans and collagen [4, 5, 6] as well as striated muscle fibers [3, 4, 7].

The cellular mechanisms underlying inguinoscrotal testis descent remain unclear. Active cell processes occurring in the gubernaculum during its size reduction may be involved: cell proliferation, differentiation and migration [8, 9, 10]. Furthermore, since the gubernaculum pulls down the testis by reducing its size, some authors have suggested gubernacular muscle contraction as the main process for testis descent [7, 11]. However, others consider that active regulation of the gubernacular involution underlies inguinoscrotal testis descent [3, 4, 12]. Whatever the reason, it is widely accepted that the inguinoscrotal phase of testis descent depends substantially on androgens [1, 2, 13, 14]. In the rat, androgen receptors (AR) are present in gubernacular cells between days 15 and 18 of gestation with a fall just before the final migration of the testicle [15, 16, 17]. Inguinoscrotal testis descent can be inhibited in animals with the postnatal administration of estradiol [18].

Although in humans with cryptorchidism, the standard form of therapy is surgical orchiopexy, hormone therapy has also been advocated. The most commonly used hormone is human chorionic gonadotrophin (hCG). The latter therapy, however, remains contro-

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versial since the direct or indirect effects of exogenous hormones on tissues forming the gubernaculum during testis descent are poorly known. The aim of this study was to correlate the location of the cells expressing AR with the ultrastructural changes occurring in the gubernaculum induced by hCG treatment in rats.

Materials and methods

Animals

All animals were treated according to the ethical principles and regulations specified by the Animal Care and Use Committee of our institution and the standards established by the National Institutes of Health of Mexico (Mexicana-NOM-062-ZOO-1999: "Technical specifications for production, care and use of laboratory animals." D.O.F. 22-VIII-2001).

Cryptorchidism was induced in Wistar rats treated daily for 30 days with subcutaneous injections of 0.1 μg of 17-β-estradiol (E2) (Sigma, Mexico City) in 50 μl of propylene glycol. The treatment was started 3 days postpartum (dpp) according to Lein et al. [18]. Thereafter, seven E2 treated rats were killed at 33 dpp and 1.0 ml of blood plasma was taken for radioimmunoassay (RIA). The remaining cryptorchid rats were divided in two groups containing 12 animals each. The cryptorchid hCG group was treated daily from 33 to 43 dpp with subcutaneous injections of 25 IU/kg body weight of hCG (Sigma) in H_20 as the vehicle. The other group was the cryptorchid control group treated with H_20. In each group, 12 animals were used for AR detection. In each of the latter groups, six rats were killed at 38 dpp and six others at 43 dpp.

Testosterone radioimmunoassay

Blood plasma samples were taken from each group at 38 and 43 dpp. RAI of testosterone was performed using a kit obtained from Diagnostic Products (Los Angeles, Calif., USA). This assay detects over 0.04 ng of testosterone/ml in plasma. Cross-reactivity with dihydrotestosterone was $<5\%$ with intra- and inter-assay variability coefficients equal to 6.5% and 10%, respectively. A one tailed analysis of variance (ANOVA) was conducted and $P<0.05$ were considered significant.

DAPI and androgen receptor immunocytochemistry

Rats were anesthetized and transcardiac perfusion was performed using 50 ml of phosphate buffered saline (PBS), followed by 100 ml of 4% paraformaldehyde. The skin from the inguinoscrotal regions was removed, and the gubernacula were excised at the base of the projections from the abdominal wall and fixed in paraformaldehyde for 30 min. After washing the samples in PBS, they were stored for 2 days in PBS containing 30% sucrose at 4°C. The gubernacula were then were embedded in OCT medium (Tissue-Tek, Sakura Finetek, USA) and frozen at 70°C (dry ice hexane). Serial sections of 15 μm thickness were obtained using a cryomicrotome, placed on slides coated with poly-L-lysine (Sigma) and air dried. Sections were stained with DAPI (1 μg/ml), rinsed in PBS and treated with 1% Triton X-100. After blocking with 1% bovine serum albumin (BSA), sections were incubated overnight with rabbit polyclonal antibody against AR (Santa Cruz Biotechnology, USA) diluted 1:1000 in BSA. After rinsing in PBS, the sections were incubated in anti-rabbit IgG conjugated with rhodamine or FITC (Sigma), diluted 1:250 with BSA, washed in PBS and embedded in DAKO fluorescent mounting medium (DAKO, USA). Cells expressing AR in the gubernaculum were identified by the fluorescent colocalization of red (rhodamine) with blue (DAPI) colors within the nucleus of the same cell. Similarly treated sections of prostate placed on the same slide as sections the gubernaculum were used as positive controls for the specificity of the AR antibody. Negative controls were incubated without the primary antibody.

Electron microscopy

Tissues were fixed in Karnovsky solution [19] without Ca^{2+} and post-fixed in 1% OsO_4 (Merck, Darmstadt, Germany) in Zetterqvist buffer [20]. Tissue samples were washed in cacodylate buffer, dehydrated in increasing concentrations of ethanol (50–100%), propylene oxide and embedded in Epon 812 (Electron Microscopy Science, Fort Washington, Philadelphia, USA). Sections (60–70 nm) were cut with an ultramicrotome (Reichtert Ultracut S), stained with uranyl acetate and lead hydroxide and examined under a Jeol 1010 electron microscope. For light microscopy, semithin sections (1 μm) were stained with toluidine blue.

Results

Cryptorchidism was successfully induced in all rats at 33 dpp, after 30 days of E2 treatment. While 100% of animals treated with hCG showed testicular descent at 43 dpp after 10 days of treatment, untreated controls remained cryptorchid at this age. Testosterone was undetected in all cryptorchid rats at day 33 after birth when treatment with hCG was started. Levels of plasma testosterone gradually increased in rats treated with hCG; after 5 and 10 days of treatment, the mean ($\pm$SE) levels of testosterone were 2.2 ± 0.3 and 3.3 ± 0.4, respectively. On the other hand, although testosterone remained undetectable in control rats at 38 and 43 dpp, they showed a similar growth rate to those treated with hCG (results not shown).

Immunocytochemical detection of AR in the gubernaculum of E2 induced cryptorchid rats revealed clear

differences depending on the presence or absence of hCG treatment. While cells with AR-positive nuclei were abundant in hCG treated animals undergoing testis descent (Fig. 1A), they were rarely seen in the gubernaculum of untreated rats that remained cryptorchid (Fig. 1B). AR-positive cells were present along the gubernaculum of hCG treated rats 5 and 10 days after treatment. Although in the current study a quantitative analysis was not attempted, the number of AR-positive cells in the gubernaculum appeared greater at 38 dpp (5 days hCG) than at 43 dpp (10 days hCG). AR appeared to be located within the nuclei in AR-positive gubernacular cells as in sections of prostatic epithelial cells placed on the same slide which were used as a positive control (image not shown).

By counterstaining the nuclei of all the cells with DAPI (Fig. 1C), it was possible to distinguish the location of cells with AR-positive nuclei (Fig. 1D) among the different tissues that form the gubernaculum (Fig. 1E). Although scattered AR-positive cells were found in the gubernacular cord formed by fibroblasts and smooth muscle fibers, most cells with AR-positive nuclei were concentrated in the bulb. As confirmed by electron microscopy, the bulb region is formed by a layer of fibrous connective tissue and a layer formed by striated muscle fibers. Most cells in the layer of fibrous connective tissue had AR-positive nuclei while in the layer rich in striated muscle, muscle fiber nuclei appeared AR-negative (Fig. 1E).

The ultrastructural study of the gubernaculum showed similar types of cells at 38 and 43 dpp in both the cryptorchid hCG and cryptorchid control animals. In addition to collagen fibers, abundant smooth muscle cells and some fibroblasts appeared in the gubernacular cord (Fig. 2A). Since in this region smooth muscle nuclei predominate over fibroblast nuclei, the scant number of

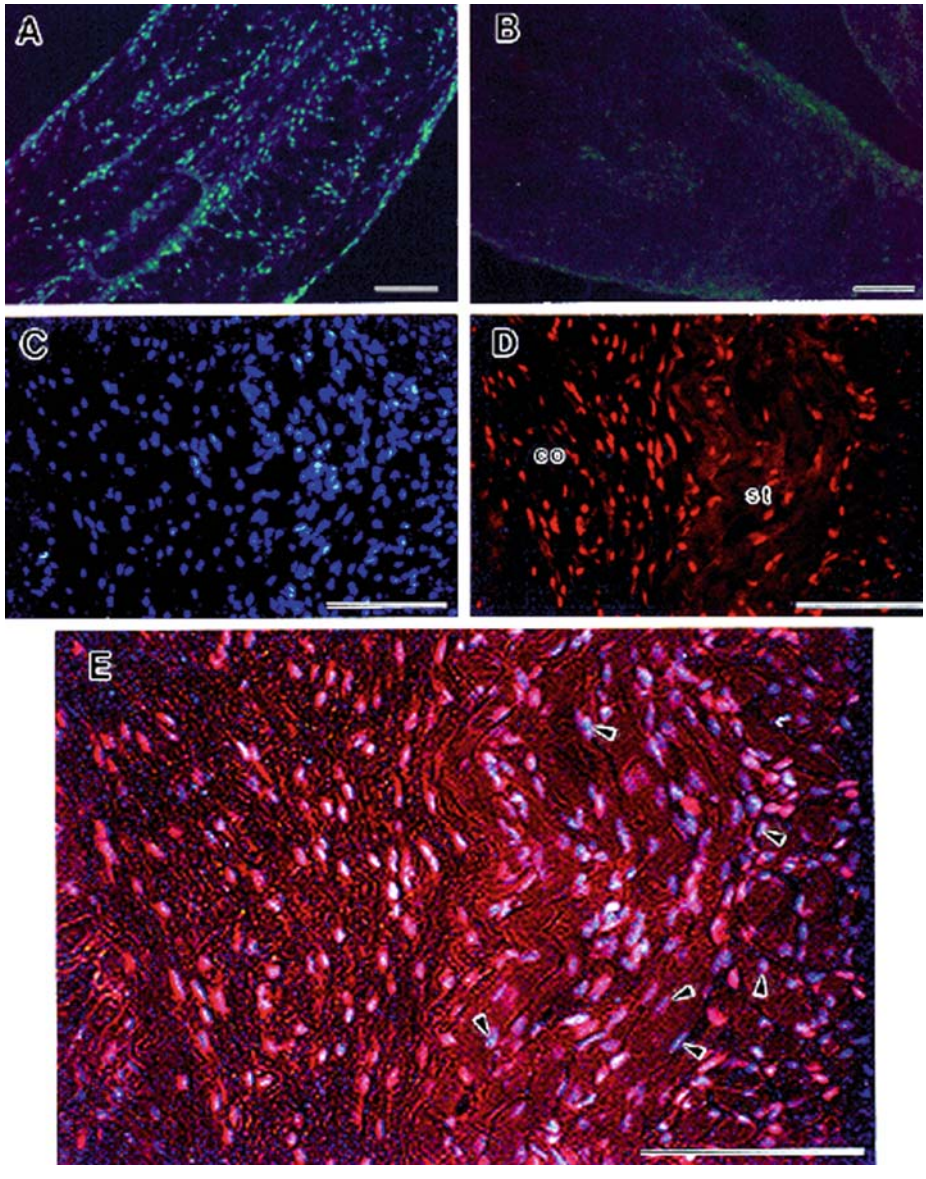

Fig. 1 Immunocytochemical detection of the androgen receptor in frozen sections of rat gubernacula dissected on day 43 after birth. **A** Shows the gubernaculum of a rat which was treated with hCG for 10 days. Notice the higher number of AR-positive cells with green nuclei compared to the gubernaculum shown in **B** corresponding to that of a control rat. **C**–**E** Show different images of the same section of the gubernacular bulb of a rat treated for 10 days with hCG. **C** Shows the nuclei of all cells stained blue with DAPI. **D** Here, only the AR-positive nuclei are stained red. The connective layer (*co*) is at the left while the layer rich in striated muscle fibers (*st*) appears at the right of this figure. **E** Pink stained nuclei indicate the colocalization of the red and blue colors shown in **C** and **D**. Notice that several nuclei of the muscle fibers are blue (*arrowheads*) suggesting their lack of AR. *Bars* = 100 μm

cells with AR-positive nuclei seen in frozen sections with the light microscope probably correspond to fibroblasts. In contrast to the cord, smooth muscle cells were rare in the bulb while fibroblasts and striated muscle fibers were abundant. Fibroblast cells were concentrated in the layer of dense connective tissue where collagen fibers were abundant (not shown). In the layer formed mainly by striated muscle cells, fibroblasts were also tightly arranged as narrow strips of connective tissue between bundles of striated muscle cells (Fig. 2B). Considering the correlation between the number and distribution of AR-positive nuclei and fibroblasts in both the cord and the bulb, it is likely that cells with AR-positive nuclei correspond to fibroblasts rather than to smooth or striated muscle cells.

Alternated semi-thin and thin sections of both the cord and the bulb of hCG treated rats showed conspicuous clusters of electron dense amorphous material in the extracellular matrix which were absent in the cryptorchid controls. The clusters of amorphous material were randomly distributed among dense bundles of collagen fibers in the near elongated cytoplasmic processes of fibroblasts (Figs. 2C). Furthermore, as in control animals, apoptotic cells were rarely found in the regressing gubernaculum of hCG treated rats undergoing testis descent.

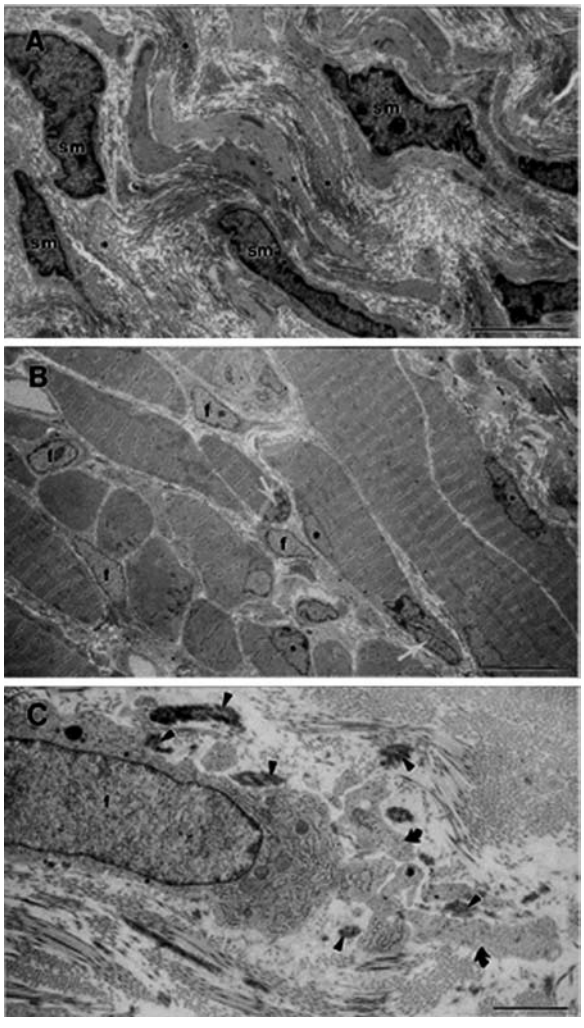

Fig. 2 Electron micrographs illustrating the ultrastructure of the gubernaculum from: **A** one control, and **B** and **C** two hCG treated animals. **A** In the gubernacular cord, the smooth muscle cells (*sm*) embedded in a collagen matrix (*asterisk*) predominate over the fibroblasts (not shown here). *Bar* = 6 μm. **B** Nuclei of satellite cells (*white arrows*), striated fibers (*asterisk*) and fibroblasts (*f*) are shown. *Bar* = 15 μm. **C**)Part of a fibroblast (*f*) located in the connective layer of the bulb showing irregular cytoplasmic processes (*arrows*). Notice several electron dense bodies (*arrowheads*) scattered in the extracellular matrix. *Bar* = 2 μm

Discussion

While during gestation abundant mesenchymal cells were found in the rat gubernaculum [3], such cells were only occasionally seen in it after birth. This finding suggests that most mesenchymal cells present in the fetal gubernaculum differentiate as fibroblasts, smooth and striated muscle cells after birth. Therefore, present observations indicate that factors required for the differentiation of mesenchymal cells into fibroblasts act during the perinatal period. The differentiation of mesenchymal cells into smooth and striated muscle fibers, however, may still take place during testis descent.

Involution of the gubernaculum during inguinoscrotal testis descent implies a size reduction. Although studies in other species suggest that regression of the connective tissue takes place during testis descent [3, 4, 12], the current results indicate that regression occurs without cell death. Apoptotic cells were rare in rats treated with hCG when testes were actively descending.

In the present study, a large number of AR-positive cells were found in postnatal rats during testis descent in estradiol treated cryptorchid rats. This is in contrast with others reports in which, during normal testis descent, a large number of AR-positive cells were seen in the gubernaculum of fetal rats but few such cells were formed after birth [15, 16, 17]. These conflicting results may be explained by the accelerated process of testicular descent induced by hCG treatment in the present experimental conditions. While in the current study inguinoscrotal testis descent lasted 10 days, normal testicular descent lasts around 21 days. Thus, a larger number of AR positive cells may be needed to carry out the same process in a shorter period of time.

In the current study, the use of DAPI, an ubiquitous nuclear stain, allowed us to determine that the cells immunoreactive to AR in the gubernaculum corresponded mainly to fibroblast cells found in great number in the hCG treated group. Furthermore, it is tempting to speculate that the amorphous material observed with the electron microscope in hCG treated rats may be collagen and other matrix glycoconjugates in the process of being digested. Testosterone stimulated fibroblasts may secrete metalloproteases involved in the involution of the

gubernacular intercellular matrix. This idea is supported by the presence of high acid phosphatase activity found in the gubernaculum during testis descent [21]. Husmann and Levy [1] proposed that androgens alter the viscoelastic properties of the gubernaculum reducing its turgidity and pushing the testis into the scrotum. In the absence of androgens, the gubernaculum becomes rigid preventing testicular descent [22]. In the current study, the process of inguinoscrotal testicular descent induced with hCG correlates with increasing levels of plasma testosterone as shown previously [23]. Testosterone acts directly or indirectly on the gubernaculum inducing testicular descent. In addition to an indirect action via the genitofemoral nerve [24], it has been proposed that testosterone acts directly on the gubernaculum inducing functional and/or morphological changes leading the testis to migrate towards the scrotum. Thus, muscle contraction and/or connective tissue remodeling may be the mechanisms underlying the gubernacular function for testis descent [3, 4, 7, 11, 12].

The current results suggest that testosterone induces the expression of AR in gubernacular fibroblasts, which in turn may degrade the extracellular matrix during gubernacular involution, leading to testis descent. The ultrastructure of the gubernaculum showed abundant striated and smooth muscle fibers located in the bulb and the cord, respectively. The presence of the two kinds of muscle fibers in the gubernaculum suggests their participation in the process of testis descent induced by hCG. According to our results, the role of testosterone in inducing smooth and striated muscle contraction for testis descent appears to be complex. Since our current results show that the AR were mainly located in connective cell nuclei and not in muscle fiber nuclei, at least two possible mechanisms can be postulated: (1) testosterone action is mediated by fibroblasts which contain AR by changing the conditions of the intercellular matrix that leads to gubernacular muscle contraction, and (2) testosterone induces gubernacular muscle contraction through a non-genomic pathway [25].

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