Morphological changes induced by testosterone in the mammary glands of female Wistar rats

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Abstract

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Increased levels of androgens in postmenopausal women are considered to be a risk factor for breast cancer. Testosterone, alone or in combination with estrogen, induces epithelial dysplasia and mammary tumors in Noble rats. Since this model of hormone-induced neoplasia has not been reported in other rat strains, we studied the effect of testosterone on the mammary gland morphology of female Wistar rats. Sixty adult, non-castrated, female Wistar rats were implanted in the dorsum midline with a silicone tube containing 50 mg testosterone (testosterone propionate in 30 animals and non-esterified testosterone in the remaining 30 animals) and 20 additional animals were implanted with empty tubes and used as control. Five animals per group were killed 30, 60, 90, 120, 150, and 180 days after implantation, and the mammary glands were dissected, fixed and embedded in paraffin. Histological sections were then stained with hematoxylin and eosin and picrosyrius red for collagen visualization. Morphological and morphometric analysis demonstrated ductal proliferation and acinotubular differentiation with secretory activity in all treated animals, peaking at 90 days of androgen exposure. After 90 days the proliferation of acinar epithelial cells was evident, but there was a progressive reduction of secretory differentiation and an increase in intralobular collagen fibers. There was no morphological evidence of dysplastic changes or other pre-neoplastic lesions. Testosterone treatment applied to adult, non-castrated female Wistar rats induced a mammary gland hyperplasia resembling the lactating differentiation, with progressive reduction in secretory differentiation.

Introduction

Since increased androgen levels are observed in postmenopausal women and are even higher in those with breast cancer, these androgens have been proposed to be carcinogenic for mammary glands (1-3). In spite of strong evidence of association between androgens and mammary cancer, there is no direct conclusive evidence indicating that this association is a cause or an effect of tumor development (4,5). In contrast to the effect of androgens increasing mammary carcinogenesis, an inhibitory action of andro-

gens on different lineages of mammary cancer cells has been reported (reviewed in Ref. 6).

Experimental data are conflicting, with reports of a high frequency of mammary cancer in adult female Noble rats treated with testosterone and estrogen (7,8). Treatment with testosterone alone also induced mammary tumors in the same rat strain (7,8). In contrast, inhibitory effects of androgens on the development (9) or progression (10) of dimethylbenz(a)anthracene-induced mammary tumors in female rats have been reported. Occasional enhancement of tumor growth was observed in some experiments in which moderate or very high doses of androgen were used (reviewed in Ref. 11).

Ductal proliferation with development of acinotubular structures has been reported in mammary glands of female rats or mice treated with testosterone soon after birth (12) and near or after sexual maturation (13). Similar effects have been reported in castrated female rats after long-term treatment with the androgen precursors dehydroepiandrosterone and dihydrotestosterone (14).

Since androgens have been used for hormonal replacement therapy in postmenopausal women (15-17) and since these hormones have been proposed to be potential carcinogens or promoters of carcinogenesis in Noble rats (7,8), but not in other rat strains, we studied the changes induced by testosterone in the mammary glands of adult female Wistar rats to determine if this hormone was carcinogenic.

Material and Methods

Eighty adult female Wistar rats aged 90-95 days housed in groups of 10 per cage were allowed free access to tap water and a commercial pellet diet. The experiments were conducted at the Laboratory of Experimental Pathology, Biomedical Center, Federal University of Espírito Santo, Vitória, ES, Brazil.

The rats were divided at random into two groups as follows: a) 60 rats received implants (a 35 x 2-mm silicone tube) with 50 mg of non-esterified testosterone (30 rats) or testosterone propionate (30 rats); b) 20 rats received empty implants and were used as control. Esterified and free testosterone (powder; Akzo-Nobel, Arnhem, The Netherlands) were used because the esterified form is inactivated slowly, and both have been used in hormonal replacement therapy. In all rats the implants were inserted subcutaneously in the dorsum midline.

At 30, 60, 90, 120, 150, and 180 days post-implant groups of 5 rats from each testosterone group and 3 rats from the control group were killed after pentobarbital anesthesia. The mammary glands were carefully excised, fixed in Bouin fluid (diluted 1:4 in 0.15 M NaCl) and embedded in paraffin. Sections were stained with hematoxylin and eosin and by the picrosirius red method for collagen. The morphological study focused mainly on the epithelial component of the mammary glands (acini and ducts) and on fibrous tissue within the lobules. Morphometric analysis was performed by the point-counting method (18) on five random photomicrographs taken with a 10X objective and printed at the same magnification. A transparent grid was placed on the photomicrographs and the crossing points of the grid over the ducts or acini, adipose tissue and fibrous stroma were counted. The results are reported as volume density (%) of glands (acinotubular structures) in relation to the total volume of all the structures of the mammary gland (18) (volume density of acinotubular structures = points counted over acinotubular structures/points counted over acini, ducts, adipose tissue, and fibrous stroma x 100). Because the abdominal gland was easier to identify correctly (presence of two lymphatic nodules) it was used for morphometric analysis.

The quantitative results were compared by the Kruskal-Wallis test and the differ-

ences were considered to be significant for P values of less than 0.05.

Results

All the implants retrieved after 180 days contained small amounts of the hormone, demonstrating the continuous release of the drug during the experiment.

The rats treated with testosterone showed normal weight gain during the experiment and developed hypertrophy of the clitoris and more aggressive behavior than the control group (data not shown).

The morphological alterations observed were similar in rats treated with testosterone propionate or non-esterified testosterone. Microscopic examination showed proliferation of terminal and lateral end buds and acinotubular differentiation in all treated animals after 30 days of treatment. In all lobules there was secretory activity, with dilatation of acini and ducts and accumulation of secretion in the lumen (Figure 1B and C). The proliferation and secretory activity peaked at 90 days of androgen exposure, as demonstrated by morphologic observation and by the volume density of mammary glands obtained by morphometric analysis (Figure 2). After 90 days the glands presented areas with different glandular patterns, with some showing acinotubular structures with typical secretory activity and others showing less evident secretory differentiation, with reduction of the secretion accumulated in the lumen. Although the volume density of acinotubular structures did not increase, the acinar cells proliferated, filling the lumen. Several acinotubular structures showed acini with a solid pattern, filled with epithelial cells with small, clear vacuoles in the cytoplasm. The solid patterns of the acini as well as the reduction in secretory differentiation were progressive through 120, 150, and 180 days of androgen exposure (Figure 1). In all rats killed at 180 days the acinotubular structure had vacuolated cells, but aci-
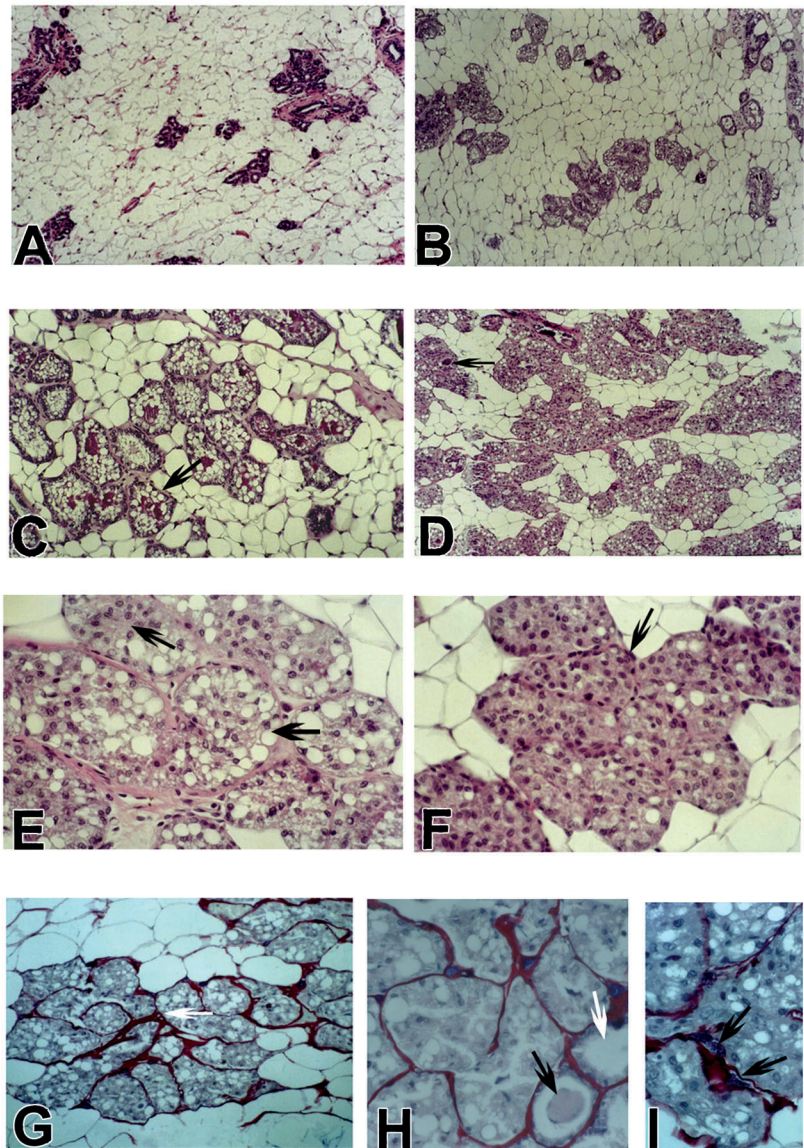

Figure 1. Different morphologic aspects of the mammary gland after 6-month exposure of female rats to testosterone. *A*, Low power view of the mammary gland of a control rat (100X). *B*, Testosterone, 30 days. Acinotubular differentiation with secretory activity (100X). *C*, Testosterone 60 days. Dilated acini (arrow) with secretory activity and with secretion in the lumen (hematoxylin and eosin, H&E, 200X). *D*, Testosterone 120 days. Persistence of the acinotubular hyperplasia, with the acinar cells filling the acinar lumen. Few acini (arrow) with secretion accumulated in the lumen (H&E, 200X). *E*, Acini with vacuolated (secretory) epithelial cells filling the lumen (arrows). In some acini there is loss of secretory differentiation evidenced by the absence of vacuoles. The nuclear pattern is not altered: there are dark and clear nuclei (H&E, 400X) *F*, Testosterone 180 days. Acini with epithelial cells filling the lumen, without vacuolization (arrow). In some acini there are vacuolated epithelial cells evidencing persistent secretory activity (H&E, 400X). *G*, *H* and *I*, Testosterone 180 days. Picrosirius red and hematoxylin staining for collagen. In *G* the white arrow indicates increased intralobular collagen stained in red by sirius red. Epithelial cells with and without secretory activity are filling the acini (200X). In *H* some acini are dilated, with (black arrow) or without (white arrow) secretory material in the lumen and with hypotrophic acinar epithelial cells (400X). In *I* the arrows indicate prominent basophilic mast cells stained by hematoxylin (400X).

nar epithelial cells without secretory activity were frequently present (Figure 1D-F). Most of the acini presented a solid pattern, with vacuolated acinar cells filling the lumen. The nuclear pattern observed in proliferating acinar cells, with dark and clear nuclei, was similar to that observed in normal mammary acini. Epithelial proliferation filling the duct lumen was not observed and the myoepithelial cells apparently were unaffected. There was increased intralobular deposition of collagen after 90 days of androgen exposure (Figure 1H). Mast cells were frequently prominent in the intralobular stroma with basophilic granules in the cytoplasm (Figure 1I).

The proliferation and secretory effects induced by testosterone exposure were similar in all mammary gland pairs. No dysplastic lesions with nuclear pleomorphism or neoplastic lesions were observed in any treated animal.

Discussion

Adult, non-castrated, female rats were used because androgens have been used in combination with estrogens in hormonal replacement therapy during perimenopause (6), when the ovaries still have some degree of activity.

The present study clearly demonstrated the potent proliferative effect of testosterone on the mammary glands of adult, non-castrated, female Wistar rats. There was ductal hyperplasia with differentiation of acinotubular structures and prominent secretory activity. Our observations up to 90 days of

exposure are similar to those reported for non-castrated adult female rats treated with testosterone propionate for 90 days (13) or castrated female rats treated with dehydroepiandrosterone for 12 months (14). The histomorphologic pattern observed up to 60-90 days of testosterone exposure resembles those seen during pregnancy and lactation and could be defined as pseudo-gestational hyperplasia. After 90 days there was a progressive loss of secretory differentiation, with increased deposition of collagen in the intralobular stroma. However, epithelial hyperplasia was not seen in the ducts and nuclear pleomorphism was not observed in the acinar or ductal epithelial cells. In addition, proliferative lesions resembling those described by Xie et al. (7,8) in female Noble rats treated with testosterone were not observed. Thus, it appears that testosterone, although it induces a secretory acinotubular hyperplasia in non-castrated female Wistar rats, does not induce apparent preneoplastic lesions after 6 months of hormonal treatment.

The progressive loss of secretory differentiation after 90 days of testosterone treatment has not been reported by other investigators who studied the effects of testosterone on non-castrated adult female rats. This may be due to differences in the time when treatment was started (11) or to the short period of observation (13). Sourla et al. (14) did not report alterations in the secretory pattern of the mammary acini of castrated rats up to 12 months of treatment with dehydroepiandrosterone.

We have no explanation for the effect of testosterone on the mammary gland, inducing a strong proliferative and secretory activity, followed by a reduction in secretory differentiation. Clinical observations in women indicate that testosterone induces atrophy of the mammary gland. However, in experimental animals (rats and mice) the hormone induces acinotubular differentiation and hyperplasia. In castrated female rats

Figure 2. Volume density of the mammary gland (acini and ducts) of adult female rats that received a silicone implant with 50 mg of testosterone. Co = control group consisting of 120-day-old female adult rats that received an empty silicone tube. The animals were killed 30, 60, 90, 120, and 180 days after implant of a silicone tube with testosterone. Data are reported as the mean ± SD for five animals per group. *P < 0.05 compared to the control group (Kruskal-Wallis test). +P < 0.05 compared to 30 or 60 days (Kruskal-Wallis test). No significant differences were observed between the groups sacrificed at 90, 120, and 180 days (Kruskal-Wallis test).
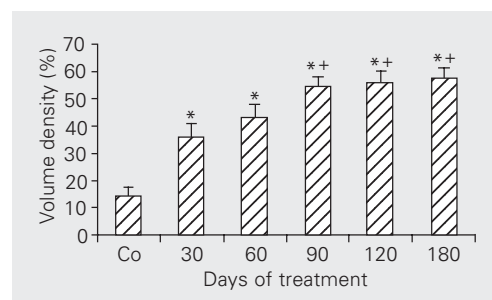
the induction of mammary acinotubular proliferation and secretion was blocked by flutamine, indicating a direct action of testosterone on androgen receptors in acinar and tubular cells (14). In non-castrated female rats the presence of an intact ovary would modify some effects of testosterone because this hormone induces increased expression of prolactin receptors in mammary epithelial cells (19). In addition, the proliferative effect of exogenous testosterone may be related to the increased estrogen levels as a consequence of two other possible mechanisms: i) mammary glands have a high aromatase activity that transforms testosterone to estrogen (6); ii) corpus luteum was not observed in the ovaries of androgen-treated rats (data not shown), that probably were not cycling, with persistent production of estrogen in follicles. However, in these experiments plasma estrogen levels were not determined in testosterone-treated rats.

The increased collagen deposition in the intralobular stroma has not been reported in castrated female rats treated with dihydrotestosterone or dehydroepiandrosterone after 12 months of exposure (14) or in non-castrated animals after 90 days of treatment with testosterone propionate (13). Only Xie et al. (7,8) reported a proliferative effect of androgens on mammary periacinar connective tissue of Noble rats, and these investigators proposed that the carcinogenic effect of testosterone may be mediated by paracrine interrelations between stromal and epithelial cells.

Our data demonstrate that long-term testosterone treatment of non-castrated, adult, female Wistar rats induced proliferation and secretory activity of the mammary glands with a progressive loss of secretory differentiation after 120 days, a result that could be defined as pseudo-gestational hyperplasia. The lack of evidence of epithelial dysplasia, as reported by Xie et al. (7,8), may reflect the differences in expression of androgens and estrogen receptors between Noble and Wistar rats (8,20,21).

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