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Testosterone inhibits estrogen/progestogen-induced breast cell proliferation in postmenopausal women

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ABSTRACT

Objective: During the past few years serious concern has been raised about the safety of combined estrogen/progestogen hormone therapy, in particular about its effects on the breast. Several observations suggest that androgens may counteract the proliferative effects of estrogen and progestogen in the mammary gland. Thus, we aimed to study the effects of testosterone addition on breast cell proliferation during postmenopausal estrogen/progestogen therapy.

Design: We conducted a 6-month prospective, randomized, double-blind, placebo-controlled study. A total of 99 postmenopausal women were given continuous combined estradiol 2 mg/norethisterone acetate 1 mg and were equally randomly assigned to receive additional treatment with either a testosterone patch releasing 300 μg/24 hours or a placebo patch. Breast cells were collected by fine needle aspiration biopsy at baseline and after 6 months, and the main outcome measure was the percentage of proliferating breast cells positively stained by the Ki-67/MIB-1 antibody.

Results: A total of 88 women, 47 receiving active treatment and 41 in the placebo group, completed the study. In the placebo group there was a more than fivefold increase ($P < 0.001$) in total breast cell proliferation from baseline (median 1.1%) to 6 months (median 6.2%). During testosterone addition, no significant increase was recorded (1.6% vs 2.0%). The different effects of the two treatments were apparent in both epithelial and stromal cells.

Conclusions: Addition of testosterone may counteract breast cell proliferation as induced by estrogen/progestogen therapy in postmenopausal women.

Key Words: Hormone therapy – Testosterone treatment – Breast cell proliferation.

During the past few years serious concern has been raised about the long-term safety of combined estrogen/progestogen hormone therapy (EPT), in particular about the effects on the breast. Clinical and observational studies have revealed an increased risk of breast cancer among postmenopausal women during such treatment.[1,2] The basis of risk associated with hormonal therapies may lie in the regulation of cell proliferation.[3] Within populations of cells in vitro and in vivo, a high rate of cell proliferation may increase the risk of transformation into the neoplastic phenotype. We previously reported a three- to fivefold increase in breast cell proliferation as assessed in fine needle aspiration (FNA) biopsy specimens among postmenopausal women receiving combined EPT.[4,5] There is a need to define new principles for treatment that are safe and to explore mechanisms to counteract adverse breast cell stimulation during treatment.

Several observations suggest that androgens may counteract the proliferative effects of estrogen and

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progestogen in the mammary gland. In cell cultures and animal experiments, androgens have been shown to exert antiproliferative effects.[6-8] We have also demonstrated a negative association between breast cell proliferation and serum levels of free testosterone in both pre- and postmenopausal women.[5,9] Furthermore, treatment with tibolone, a compound having androgenic properties, did not increase breast cell proliferation, as shown with conventional EPT.[5]

Human studies on the effects of testosterone on the breast have been hampered by the lack of preparations suitable for women. However, a transdermal patch releasing 300 μg of testosterone per day is currently being evaluated in clinical trials in postmenopausal women.[10]

So far, no prospective, randomized, controlled studies on the effects of exogenous testosterone on the breast have been performed. We now report a prospective, randomized, double-blind, placebo-controlled trial on the effects of testosterone addition on breast cell proliferation in postmenopausal women undergoing continuous combined EPT.

METHODS

Participants

This study was conducted at the Women's Health Research Unit at the Karolinska University Hospital, Stockholm, Sweden. Naturally postmenopausal, apparently healthy women aged 45 to 65 years with a body mass index of more than 18 and less than 30 kg/m^2 were recruited for the study. They had all been postmenopausal for at least 12 months and had follicle-stimulating hormone levels greater than 40 IU/L. None of the women had taken any sex steroid hormones during the last 3 months before the study. All the women had a normal mammogram within 1 month of entering the study. Exclusion criteria were any previous history of cancer, any history of previous breast disease or an abnormal mammogram, hypertension (systolic blood pressure >170 mm Hg or diastolic >105 mm Hg), hyperlipidemia (total cholesterol >8.0 mmol/L or triglycerides >3.0 mmol/L), diabetes mellitus, a history of thromboembolic disease, undiagnosed vaginal bleeding, any sign of hepatic dysfunction, or concomitant treatment known to influence the study medication (warfarin, rifampicin, carbamazepine, griseofulvin, hydantoins, primidone, barbiturates, and broad-spectrum antibiotics). The study was approved by the Independent Ethics Committee at the Karolinska University Hospital (IRB project 02-217) and by the Swedish Medical Products Agency (151:2002/40281). All women gave their written informed consent before inclusion.

Study objectives

The primary objective of this study was to investigate the effects of testosterone when added to continuous combined EPT on breast cell proliferation. The main outcome measure was the percentage of proliferating cells that were positively stained by the Ki-67/MIB-1 monoclonal antibody.

Study design

A total of 99 women were given continuous combined oral treatment with 17β-estradiol (E_2) 2 mg and norethisterone acetate (NETA) 1 mg (Kliogest, Novo Nordisk A/S, Copenhagen, Denmark) once daily for 6 months. In addition, they were randomly assigned in a 1:1 ratio to receive either a testosterone patch releasing 300 μg/24 hours (testosterone transdermal system, Procter & Gamble Watson Laboratories, Salt Lake City, UT) or a placebo patch applied twice weekly to the abdomen. Blinding was maintained until completion of the study. Compliance and vital signs were checked at clinical visits after 2, 4, and 6 months. Physical examination, including pelvic examination and breast palpation, was carried out at baseline and after 6 months of treatment. The women were asked about any untoward medical events during the trial.

Breast cell proliferation

Before and after 6 months of treatment, percutaneous FNA biopsies from the upper outer quadrant of the left breast were performed using a needle with an outer diameter of 0.6 mm, as described by Franzen and Zajicek[11] and Skoog et al.[12] To produce several identical slides, the aspirated cells were mixed with 0.5 to 1.0 mL of 4% buffered (pH 7.4) formalin in the syringe used to procure cells. The cells were concentrated by centrifugation at 700 rpm for 3 minutes in a Shandon Cytospin centrifuge (Labex Instruments, Helsingborg, Sweden), and after resuspension in 200 μL of buffered formalin, volumes of 110 μL were sedimented onto pretreated glass slides.

Immunocytochemical analysis

Immunostained cells were quantified using cell counting. Slides were blinded for identity, type of treatment, and sequence of biopsy and stained for the nuclear antigen Ki-67. The Ki-67/MIB-1 monoclonal antibody reacts with a human nuclear antigen that is present in proliferating cells but absent in quiescent

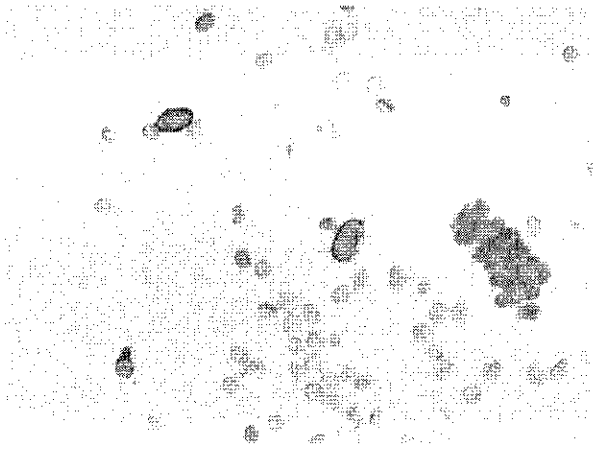

FIG. 1. Illustration of two stromal breast cells with positive staining for vimentin. Stromal cells are also morphologically separated from epithelial cells by a two- to threefold larger size and an oval shape.

cells. Cell cycle analysis shows that the antigen is expressed in the phases of G_1, S, G_2, and mitosis.[13] MIB-1 analyses were performed using reagents supplied by Immunotech (Marseilles, France). The staining procedure uses an avidin-biotin peroxidase system, modified for the Cytospin technique. We considered samples obtained by FNA to be assessable only if they contained intact cells and no free-lying nuclei. All slides were examined by an experienced cytopathologist (L.S.), and approximately 80% of cells were judged to be of epithelial origin. Stromal cells were identified morphologically by size (twice that of epithelial cells) and an oval shape and initially also by positive staining for vimentin using a commercial kit (Monoclonal Mouse Anti-Vimentin M7020, DAKO A/S, Glostrup, Denmark). Vimentin is a skeletal protein present only in cells of mesenchymal origin[14] (Fig. 1). On average 150 to 200 cells were counted per slide, and only samples with a minimum of 40 cells were included in the analysis.

Analytical methods

Venous blood samples were drawn on the day of FNA biopsy. Serum concentrations of testosterone and of E_2 were determined by radioimmunoassay using commercial kits from Diagnostic Products Corporation, Los Angeles, CA (Coat-a-Count, testosterone), and CIS Bio International, Gif-sur-Yvette, France (ESTR-US-CT, E_2). Sex hormone–binding globulin (SHBG) and insulin-like growth factor 1 (IGF-1) were determined by chemiluminescence enzyme immunoassays using commercial kits obtained from Diagnostic Products Corporation (Immulite, SHBG) and from Nichols Products Corporation, San Juan Capistrano, CA (Advantage, IGF-1). Serum levels of dihydrotestosterone (DHT) were determined by radioimmunoassay after removal of cross-reacting testosterone by oxidative cleavage of the 4-ene double bond with potassium permanganate, using a commercial kit (Diagnostic Systems Laboratories Inc, Webster, TX).

Apparent concentrations of free testosterone were calculated from values of total testosterone, SHBG, and a fixed albumin concentration of 40 g/L by successive approximation using a computer program based on an equation system derived from the law of mass action.[15]

The detection limits and within- and between -assay coefficients of variation for testosterone were 0.1 nmol/L, 6%, and 10%; for free testosterone, 6 pmol/L, 7%, and 10%; for DHT, 14 pmol/L, 4%, and 8%; for E_2, 5 pmol/L, 13%, and 18%; for SHBG, 0.2 nmol/L, 6.5%, and 8.7%; and for IGF-1, 6 μg/L, 5%, and 7%, respectively.

Statistical analysis

Differences in breast cell proliferation and serum factors within and between groups were analyzed using

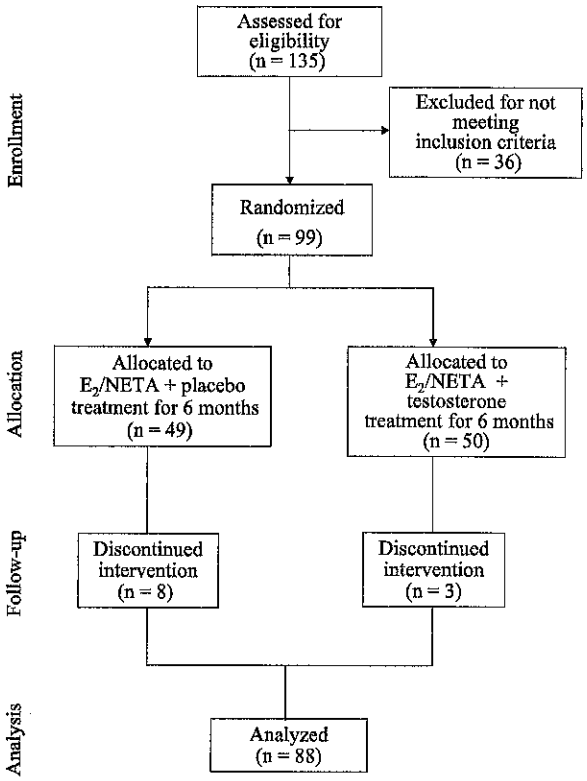

FIG. 2. Flow diagram of the randomized study. E_2, 17β-estradiol; NETA, norethisterone acetate.

TABLE 1. *Serum levels (mean ± SEM) of sex steroids, SHBG, and IGF-1 in postmenopausal women before and after treatment with E_2/NETA in combination with either placebo or a testosterone patch*

	Placebo (n = 41)		Testosterone (n = 47)	
	Baseline	6 months	Baseline	6 months
E_2 (pmol/L)	74.5 ± 12.7	253.8 ± 20.3[a]	54.7 ± 3.4	270.9 ± 19.5[a]
Testosterone (nmol/L)	0.48 ± 0.03	0.43 ± 0.02[b]	0.46 ± 0.02	1.75 ± 0.2[a]
Free testosterone (pmol/L)	8.2 ± 0.67	6.43 ± 0.4[a]	7.43 ± 0.5	26.4 ± 2.5[a]
DHT (pmol/L)	750.1 ± 61.6	717.4 ± 63.9	684.3 ± 61.6	1,462.8 ± 133.5[a]
SHBG (nmol/L)	47.5 ± 2.3	59.0 ± 2.8[a]	46.7 ± 2.8	58.8 ± 2.7[a]
IGF-1 (μg/L)	145.9 ± 7.0	134.7 ± 4.9	143.6 ± 6.1	144.6 ± 6.2

SHBG, sex hormone–binding globulin; IGF-1, insulin-like growth factor 1; E_2, 17β-estradiol; NETA, norethisterone acetate; DHT, dihydrotestosterone.
[a] $P < 0.01$.
[b] $P < 0.05$.

Wilcoxon signed-rank and Mann-Whitney *U* tests. Correlations were assessed by Spearman's rank-order correlation coefficient. A *P* value less than 0.05 was considered significant.

RESULTS

A total of 135 postmenopausal women were assessed for eligibility, and 36 did not fulfill the inclusion criteria. Of the 99 randomly assigned women, 11 were not assessable for various reasons (ie, discontinued treatment or not having FNA biopsy). Thus, a total of 88 women, 47 receiving active treatment and 41 in the placebo group, completed the study (Fig. 2).

The mean values for age and body mass index for the testosterone group were 55.2 years and 24.2 kg/m^2 and for the placebo group were 54.7 years and 25.1 kg/m^2, respectively, and did not differ between the groups. Also, no significant differences in hormonal levels were found at baseline. As illustrated in Table 1, there was a marked increase in circulating levels of E_2 in both treatment groups. Circulating levels of total and free testosterone and DHT were increased during active treatment with the patch, whereas there was a slight decline in the placebo group. The increase in SHBG levels was about the same in both groups. Although there was no significant change in the mean IGF-1 values, individual values for change showed a negative correlation with the change in SHBG ($r_s = -0.35$, $P < 0.01$) and in the testosterone group a positive association with free testosterone ($r_s = 0.31$, $P < 0.05$).

From the 88 women, a total of 176 FNA biopsy samples were obtained. Of these aspirates (75%), 132 were evaluable for MIB-1 content, 69 in the testosterone group and 63 in the placebo group; 44 biopsies (25%) were nonevaluable because of too few cells in the aspirate. The median values for total breast cell proliferation (percentage of MIB-1-positive epithelial and stromal cells) in women with assessable samples at baseline (n = 62) were 1.3% and after 6 months of treatment (n = 70) were 3.0%.

Among the 88 women, 50 (57%) had evaluable aspirates both before and after 6 months of treatment. Of these 50 women, 27 received E_2/NETA plus the testosterone patch, and 23 received E_2/NETA plus placebo. The percentages of total MIB-1-positive breast cells before and after treatment in women with two evaluable samples are given in Table 2. During treatment with E_2/NETA there was a more than fivefold increase in total cell proliferation from a median value of 1.1% at baseline to 6.2% after 6 months ($P < 0.001$). In contrast, no such increase in proliferative activity was recorded in the testosterone group (1.6% vs 2.0%). Individual breast cell samples before and after 6 months of each treatment are shown in Figure 3.

TABLE 2. *Mean value, median, and range for the total percentage of Ki-67/MIB-1-positive breast cells in 50 postmenopausal women with assessable fine needle aspiration samples, both before and after 6 months of treatment with estradiol/norethisterone acetate in combination with either placebo or a testosterone patch*

	Baseline	6 Months
Placebo		
n	23	23
Mean	1.7	5.1[a]
Median	1.1	6.2[a]
Range	0.0-8.5	0.0-9.7
Testosterone		
n	27	27
Mean	2.0	2.5[b]
Median	1.6	2.0[b]
Range	0.0-7.7	0.0-7.0

[a] $P < 0.001$.
[b] Not significant.

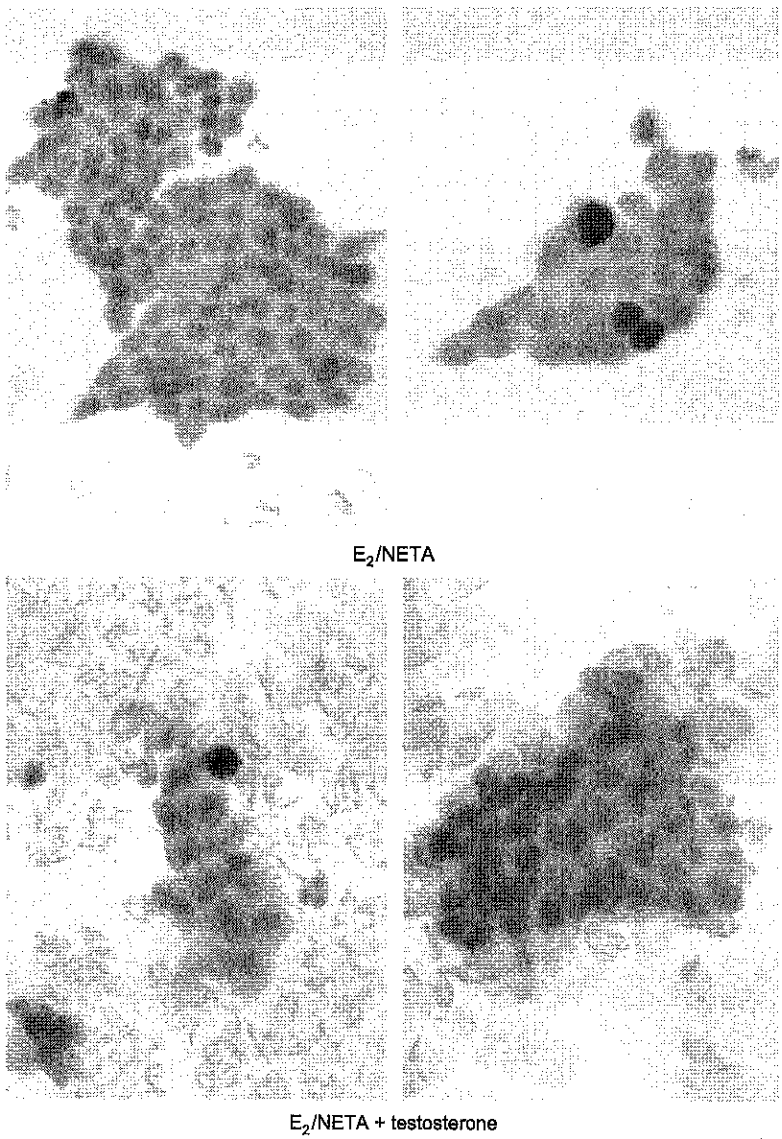

FIG. 3. Breast cells from two postmenopausal women before **(left)** and after 6 months of treatment **(right)** with 17β-estradiol (E_2)/norethisterone acetate (NETA) + a placebo patch **(top)** and E_2/NETA + a testosterone patch **(bottom)**. Nuclei in proliferating cells are stained brown by the Ki-67/MIB-1 monoclonal antibody.

In addition to total breast cell proliferation, epithelial and stromal cells were separately assessed. The different effect of the two treatments was apparent in both cell types. As illustrated in Table 3, for women with paired samples both epithelial and stromal cell proliferation was markedly increased during treatment with E_2/NETA. No significant change in proliferation occurred either in epithelial or in stromal cells during treatment with E_2/NETA in combination with the testosterone patch.

In the total material at baseline (n = 62), proliferative activity in epithelial and stromal cells displayed a significant association ($r_s = 0.48$, $P < 0.05$). Proliferation was significantly higher in stromal than in epithelial cells (2.7% vs 0.5%, $P = 0.003$). After 6 months of treatment, median values for epithelial cells in the testosterone group were 1.9% and in the placebo group were 2.4%. The corresponding figures for stromal cells were 6.5% and 10.8% ($P < 0.01$). In the total material, there was an inverse correlation between stromal cell proliferation (n = 32) and DHT levels at 6 months ($r_s = -0.40$, $P < 0.05$).

Women with two evaluable samples had significantly lower levels of DHT at baseline compared with

TABLE 3. *Mean value, median, and range for the percentage of Ki-67/MIB-1-positive epithelial and stromal breast cells in postmenopausal women with assessable fine needle aspiration samples, both before and after 6 months of treatment with estradiol/norethisterone acetate in combination with either placebo or a testosterone patch*

	Baseline	6 Months
Placebo		
Epithelial (n = 22)		
Mean	1.1	3.4[a]
Median	0.0	2.8[a]
Range	0.0-6.1	0.0-8.3
Stromal (n = 9)		
Mean	4.5	13.0[b]
Median	2.4	12.7[b]
Range	0.0-16.7	1.8-25.0
Testosterone		
Epithelial (n = 21)		
Mean	1.6	2.2[c]
Median	1.4	2.0[c]
Range	0.0-4.8	0.0-6.5
Stromal (n = 4)		
Mean	3.8	3.1[c]
Median	3.8	2.0[c]
Range	0.0-7.5	0.0-8.6

[a] $P < 0.001$.
[b] $P < 0.05$.
[c] Not significant.

women with insufficient samples (mean ± SEM, 664.7 ± 48 vs 791.1 ± 57.8 pmol/L; $P < 0.05$) but otherwise displayed no apparent differences.

DISCUSSION

This is the first prospective, randomized study on the effects of testosterone on breast cell proliferation in postmenopausal women. After 6 months of treatment with E_2/NETA, we found a marked increase in breast cell proliferation that was apparent in both epithelial and stromal cells. In contrast, when the testosterone patch was added in women receiving the same estrogen/progestogen treatment, no significant increase in breast cell proliferation was recorded.

The FNA biopsy is an established technique for the preoperative diagnosis of palpable lesions in the breast.[12] Numerous reports have shown a high correlation and reproducibility between cytological analysis of FNA biopsy samples and histological follow-up of open biopsy and surgical specimens.[16] FNA biopsies cause minimal inconvenience for the patient and can be performed repeatedly to assess the effects of different hormonal treatments in the normal breast.[4,5,9,17] As in previous studies, FNA biopsy samples were obtained from the upper outer quadrant of the left breast. This area was chosen because on average a higher amount of breast epithelium is found in this location.[18] In a previous analysis of 10 different locations in the macaque breast, there were no significant differences with respect to proliferative activity and receptor expression.[19]

In general, the postmenopausal breast has low cellularity. We have previously shown that women in whom insufficient material was obtained were on average older, had more years since menopause, and had higher body mass index compared with those with assessable samples. Here, the cell yield from FNA biopsies and the percentage of assessable samples was quite similar to that in previous studies on postmenopausal women.[4,5,20]

In previous studies using the FNA biopsy technique for assessment of proliferation by the Ki-67/MIB-1 antibody, we have repeatedly found a three- to fivefold increase in breast cell proliferation during combined EPT.[4,5,9] Here, the same increase was apparent in the placebo group during 6 months of treatment with E_2/NETA. So far, there are no data on breast cell proliferation for a longer period of follow-up than 6 months. However, we have previously shown that the increase in mammographic breast density, which is associated with increased cell proliferation, is fully established during the first few months and will not increase further during prolonged treatment with the same regimen.[21]

The present results support the concept that androgens may counteract the proliferative effect of estrogen and progestogen in the mammary gland. Previously, Dimitrakakis et al[8] found treatment with flutamide, an androgen receptor antagonist, to markedly enhance breast epithelial proliferation in normally cycling rhesus monkeys. Furthermore, in castrated animals, testosterone addition was found to inhibit breast proliferation as induced by estrogen and progestogen. Women with polycystic ovaries tend to have raised endogenous androgen levels and may also carry a lower breast cancer risk.[22] Androgen receptor dysfunction has been reported in some men with breast cancer.[23] Recently a genetic linkage was suggested between androgen receptor dysfunction and *BRCA-1* mutations.[24] Previously, in fertile women using oral contraceptives we found an inverse relationship between circulating levels of free testosterone and breast cell proliferation.[9] All these observational and animal data are in good agreement with our present results obtained from postmenopausal women in vivo. In this study, levels of testosterone during treatment showed only a moderate increase. The mean value for total testosterone of 1.75 nmol/L was well below the upper range

(3.0 nmol/L) for premenopausal women, and the highest individual value was 5.87 nmol/L. Also, levels of free testosterone ranging from 5 to 78 pmol/L were not extremely high.

Breast stroma accounts for more than 80% of the resting breast volume.[25] This supportive platform for the epithelial cells is composed of collagen, fibroblasts, endothelial cells, adipocytes, and a macromolecular network of proteoglycans. In the present study, for the first time, we assessed stromal and epithelial cell proliferation separately. Although the numbers of stromal cells detected in the aspirates were generally low, we found the two distinct cell types to respond in a quite similar manner to treatment with estrogen/progestogen alone or in combination with testosterone. In fact, the proliferative activity was seemingly even more pronounced in stromal than in epithelial cells.

The regulation of breast cell proliferation in response to sex steroid hormones is complex and incompletely understood. Part of the sex steroid action in target tissues is mediated via binding to specific intranuclear receptors. Apart from estrogen and progestogen receptors, the androgen receptor is a third member of the nuclear receptor superfamily.[26]

Estrogen receptors are found in both epithelial and stromal cells within the mammary gland.[27,28] However, most cells that proliferate in response to estrogens do not contain estrogen receptors.[29-31] Experimental data suggest that the estrogenic stimulation of epithelial growth in the mammary gland is a paracrine event and mediated via estrogen receptor–positive stromal cells.[32,33] Information about androgen receptor content in breast epithelial and stromal cells is limited. It could be that the apparent inhibitory effect of testosterone on breast epithelium as demonstrated in the present study is mediated in a similar way, ie, via a change in stromal cell signaling.

It would probably be erroneous and too simplified to conclude from the present findings that testosterone is a straightforward, antiproliferative agent on the breast. Certainly the evidence from numerous studies on the relationship between testosterone and breast cancer does not allow a single unequivocal conclusion. In fact, divergent effects concerning androgens and androgen receptors in the regulation of proliferation in breast epithelial cells and breast cancer have been reported in both in vitro and in vivo studies.[6,34] Whereas experimental data suggest that ligand activation of estrogen receptors and androgen receptors may result in opposite actions on breast cell proliferation,[35] testosterone is also a known precursor for estrogen and thus an important source of bioavailable estrogen in breast tissue. In postmenopausal women with low endogenous estrogen levels and increased adipose aromatase activity, a higher testosterone level has been associated with an increased breast cancer risk.[36]

Experiments in breast cancer cell lines suggest that testosterone and other androgens may have two distinct primary effects. Under estrogen-deprived conditions androgens, after aromatase conversion, may stimulate growth via estrogen receptor-α, and this effect can be blocked by antiestrogens. On the other hand, in the presence of estrogens, androgens will inhibit the growth-stimulating effect of estrogen. This antagonistic effect is mediated via the androgen receptor and can be blocked by antiandrogens.[37,38]

CONCLUSIONS

In summary, our results indicate that the addition of testosterone to a common estrogen/progestogen regimen has the potential to modulate the stimulatory effects of hormones on breast cell proliferation. The effects of testosterone alone on the postmenopausal breast in women not receiving simultaneous estrogen/progestogen treatment remain to be elucidated.

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