

Correlation of androgen receptors, aromatase, and 5- α reductase in the human vagina with menopausal status

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Objective: To determine whether aromatase and 5 α -reductase mRNAs are expressed in human vagina and to evaluate the presence of androgen receptors in human vaginal tissue based on age and menopausal status.

Design: Prospective study.

Setting: Specimens obtained from clinical renal urology practice.

Patient(s): Premenopausal and postmenopausal women undergoing surgery for prolapse or incontinence.

Main Outcome Measure(s): Expression of aromatase and 5 α -reductase type 1 and 2 mRNAs was evidenced by reverse transcriptase-polymerase chain reaction (RT-PCR), and the density of androgen receptors was measured by semiquantitative immunohistochemistry.

Result(s): The mRNAs for aromatase and 5 α -reductase isotypes 1 and 2 were detected in vaginal specimens. Androgen receptors were present in vaginal mucosa, submucosa, stroma, smooth muscle, and vascular endothelium. Expression was significantly greater in vaginal submucosa. A negative correlation existed between age and androgen receptor density.

Conclusion(s): Expression of genes encoding for enzymes involved in testosterone metabolism in the human vagina, as well as androgen receptor location, density, and changes with menopausal status, suggests that androgens may play a role in regulating vaginal smooth muscle and vaginal blood flow. (Fertil Steril® 2003; 79:925–31. ©2003 by American Society for Reproductive Medicine.)

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It is generally accepted that 17- β estradiol (E₂) influences physiology of vulvar and vaginal skin in women, and a significant body of evidence exists on peripheral effects of estrogen (E) on female urogenital tissue and sexual response (vaginal blood flow and lubrication) (1). Vaginal mucosa is hormonally sensitive and a decline in circulating E levels results in thinning of vaginal mucosal epithelium and atrophy of vaginal wall smooth muscle (2). There are, however, limited data on the effects of androgens on female urogenital tissue, specifically vaginal structure and function. Previous studies have described E and androgen receptors in human female genital skin in an attempt to identify the potential target cells for each hormone (3). In animal models, labia majora, labia minora, and vagina stain positive for the androgen receptor (3, 4) and vaginal epi-

thelium responds to testosterone (T) replacement in a similar manner to E replacement, even in the absence of E. Vaginal epithelium of ovariectomized mice treated with T or aromatase inhibitors have increased number of layers, thickness, and mitotic rates than controls (5).

Similar to the decline in serum E levels with aging and menopause, circulating levels of total T also decline continuously with age in women (6, 7). This is a consequence of the age-related decline in adrenal androgen production and the loss of the midcycle increase in ovarian T in the late reproductive years. After menopause, although ovarian T production continues, the total T production decreases by about 30% (8, 9). In patients undergoing oophorectomy, T levels decrease by about 50% (10). Sublingual intake of T in women causes

an increase in genital responsiveness and genital vasocongestion, 3–4.5 hours after reaching peak T level (11), in strong association with subjective reports of “genital sensations.” In contrast, low T levels (total T <20 ng/dL, free T <0.9 ng/dL) are associated with a decline in sexual arousal, genital sensation, libido, and orgasm (12). One recent study showed that transdermal T improved sexual function in women after surgically induced menopause (13). However, the precise role of T on maintaining vaginal structure, function, and sexual arousal responses is not known.

Androgen action on target organs occurs either directly through binding to androgen receptor, or indirectly through the E receptor after its aromatization. Although aromatization of T to E occurs in vaginal tissue (14), T may also have a direct effect on vaginal tissue not mediated by E. Female mice treated neonatally with large doses of androgen exhibit ovary-independent, permanent stratification and cornification on the vaginal epithelium when mature (5). Aromatase and 5 α -reductase (5 α R) levels and expression in human vaginal tissue have not been studied, although 5 α R has been found in female animal reproductive tract (15). Many women are treated with aromatase inhibitors for E-dependent breast cancer and endometriosis, although the impact on vaginal tissue is not known (16, 17). Although androgen receptors are present in the human vagina, it is unclear whether T acts as such on the receptor directly or by conversion to dihydrotestosterone.

To determine the potential route of androgen action in the vagina, aromatase and 5 α R type I and II gene expression was determined in human vaginal tissue by reverse transcriptase-polymerase chain reaction (RT-PCR) assay, and tissue localization and density of androgen receptors was assessed by immunohistochemistry. We also examined the effects of age and menopausal status on vaginal androgen receptor expression.

MATERIALS AND METHODS

Human Specimens

This study was approved by the Human Subject Protection Committee, and all discarded specimens were obtained with written informed consent. Specimens were obtained from women undergoing vaginal surgery for pelvic organ prolapse or incontinence. Thirty-one women ranging in age from 31 to 78 years (mean 62 years) were included in this study. Ten were premenopausal and 21 were postmenopausal. In the latter group, 14 were receiving oral hormone therapy (HT) in the form of E₂ with and without P (n = 3 with P and n = 11 without P). No women were on any form of T. Mean number of deliveries was 2.4. For each patient, factors including menopausal status, hormone replacement status, parity, medical history and type of previous pelvic surgery, type and degree of current prolapse (cystocele, rectocele, uterine prolapse, enterocele) were determined.

Specimens were identified by the surgeon as proximal vagina (proximal 2/3 'rds) or distal vagina (distal 1/3 'rd). Each specimen measured approximately 2 cm by 1 cm and was full thickness. The specimens were divided into two portions, one that was kept in RNA later (Ambion, Austin, TX), for RNA extraction, and the other, that was fixed in 4% formaldehyde for 16–18 hours, for immunohistochemistry.

mRNA Detection by RT-PCR

Total RNA was isolated from 50 to 100 mg of vaginal tissue using the Trizol procedure (Gibco BRL, Carlsbad, CA). Aromatase gene expression was determined by RT-PCR using “ready-to-go beads” (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). One microgram of total RNA, 0.5 μ g of random primer, and 25 pmoles of each aromatase primer were added to each tube. After the RT, samples were subjected to 45 cycles of PCR amplification (1 min each at 95°, 60°, and 72°C). The sequence of the forward primer was (5' to 3') GAATATTGGAAGGATGCACAGACT, corresponding to exon 9, and the one for the reverse primer was GGGTAAAGATCATTTCAGCATGT, complementary to a sequence of exon 10 of the human aromatase gene, with an expected product of 293 bp (18). In certain cases glyceraldehyde phosphate dehydrogenase was amplified in separate reactions as a housekeeping reference gene, using primers as previously described, with an expected product of 496 bp. The positive control was human ovary RNA (Ambion). DNA bands were visualized by ethidium bromide staining in 2% agarose gel, using appropriate molecular mass markers.

Routine RT-PCR was performed for both 5 α R-I and II isotypes, using 1 μ g of total RNA per sample, 0.5 μ g of random primer, 5 \times first strand buffer, 2 μ L of 0.1 M DTT, 10 nmoles of each dNTP, 0.5 μ L of RNase inhibitor, and 1 μ L of Superscript II. Two microliters of the RT reaction were used to perform the PCR assay using 30 cycles (1 min each at 95°, 58°, or 59°C for isotypes I or II, respectively, and 72°C). The primers sequences used for 5 α R-I were TGCTGATGACTGGGTAACAG (forward) and GTTG-GCTGCAGTTACGTATTC (reverse) with an expected product of 171 bp (19). The primers sequences used for 5 α R-II were CCTTGTACGTCGCGAAGC (forward) and 5'CCACCCA TCAGGGTATTTCAG (reverse), with an expected product of 350 bp (19). The positive controls for 5 α R-I and 5 α R-II were human prostate RNA (Ambion). DNA bands were visualized as above.

Immunohistochemistry

Fixed vaginal specimens were paraffin-embedded, and 3- μ m sections were obtained. Immunodetection was performed with a polyclonal antibody against the human androgen receptor protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Preabsorbed antibody was prepared by incubating 1 μ g/mL of androgen receptor antibody with 20 μ g of synthetic antigen peptide for 2 hours at room temperature. Negative control slides received normal mouse immunoglobulins (DAKO Corp, Carpinteria, CA) as the primary

antibody. Visualization of the immunoreaction was carried out with a secondary goat anti-rabbit IgG antibody linked to horseradish peroxidase and diaminobenzidine. Harris hematoxylin was applied as a nuclear counterstain (20).

Three slides for each specimen were examined microscopically by one trained examiner for nuclear receptor staining, and were graded by percentage of positive cells per high-power field (0 = 0%, 1 = <50%, 2 = 50%, 3 = >50%, 4 = 100%). In addition, a rating system was set for the average amount of stain intensity in cells per high-power field (0 = none, 1 = negligible, 2 = minimal, 3 = moderate, 4 = extensive). Androgen "density" scores were computed by multiplying the intensity score (0–4) by the percent of cell-stained score (0–3), where the percent of cells stained is a measure of androgen responsive cells. The observations were classified according to whether mucosal or submucosal regions were examined. Mean mucosal and submucosal androgen density scores were computed across all samples for a given woman. Mean scores for all factors were computed to obtain the factor level mean.

Statistical Analysis

For each factor investigated (vaginal location, number of vaginal deliveries, menopausal status, etc.), a one-way univariate ANOVA was used to compare mean androgen receptor density levels and compute corresponding standard errors and *P* values. For vaginal location (proximal vs. distal vagina), a random effects ANOVA model was used to compare means to account for possible correlations among multiple samples from the same woman and to be able to use the data from all samples. Model-based means and standard errors, after adjusting for any correlations, are reported. The Spearman rank correlation was used to assess the association between age and androgen receptor density.

RESULTS

mRNA Expression of Genes Related to T Metabolism

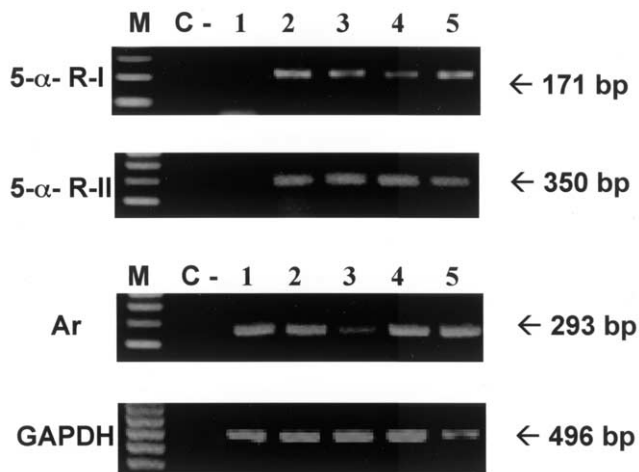
Twenty vaginal samples were randomly selected from different patients to determine gene expression for 5 α R-I and 5 α R-II, and a subset of 10 was also used for aromatase. The RT-PCR amplification demonstrated the presence of the expected 171, 350, and 293 bp bands, as well as the house-keeping gene, as shown in Figure 1. Eighteen of the 20 specimens showed expression of the 5 α R-I isoform, and 14 were positive for 5 α R-II. Two of the specimens did not express either of the two isoforms. Aromatase expression was demonstrated in 7 of 10 samples selected.

Androgen Receptor Expression and Distribution

Androgen receptors were detected by immunostaining in vaginal mucosa, submucosa, stroma, smooth muscle, and vascular endothelium of proximal and distal vaginal specimens, as shown in Figure 2. Visual quantitation of androgen

FIGURE 1

mRNAs encoding enzymes for T metabolism in vaginal tissue. RNAs were subjected to RT/PCR in separate reactions with each set of primers: 5 α R-I and 5 α R-II = 5 α -reductase-I and II; Ar = aromatase; GAPDH = glyceraldehyde phosphate dehydrogenases. M = 100 bp; C = negative control; and 1, 2, 3, 4, 5, 6, and 7 = patients' numbers.



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receptor density showed that receptor expression was significantly greater in vaginal submucosa as opposed to mucosa (*P* = .05). In addition, proximal vaginal specimens had significantly (*P* = .0008) higher mean androgen receptor density scores (3.08 ± 0.67) than distal vaginal specimens (1.47 ± 0.17).

TABLE 1

Factors significantly associated with increased vaginal mucosal and submucosal androgen receptor density.

Factor	Androgen in mucosa	Androgen in submucosa
Vaginal location (prox vs. distal)	No	Yes
Number of vaginal deliveries (1–6)	No	No
Menopausal status	No	Yes
Prior pelvic surgery (yes or no)	Yes	No
Uterine prolapse (yes or no)	No ^a	No ^a
Cystocele (no vs. grade 2 or higher)	No	No
Rectocele (no vs. grade 2 or higher)	No	No
Enterocoele (no vs. grade 2 or higher)	No	No
Age	Yes	No

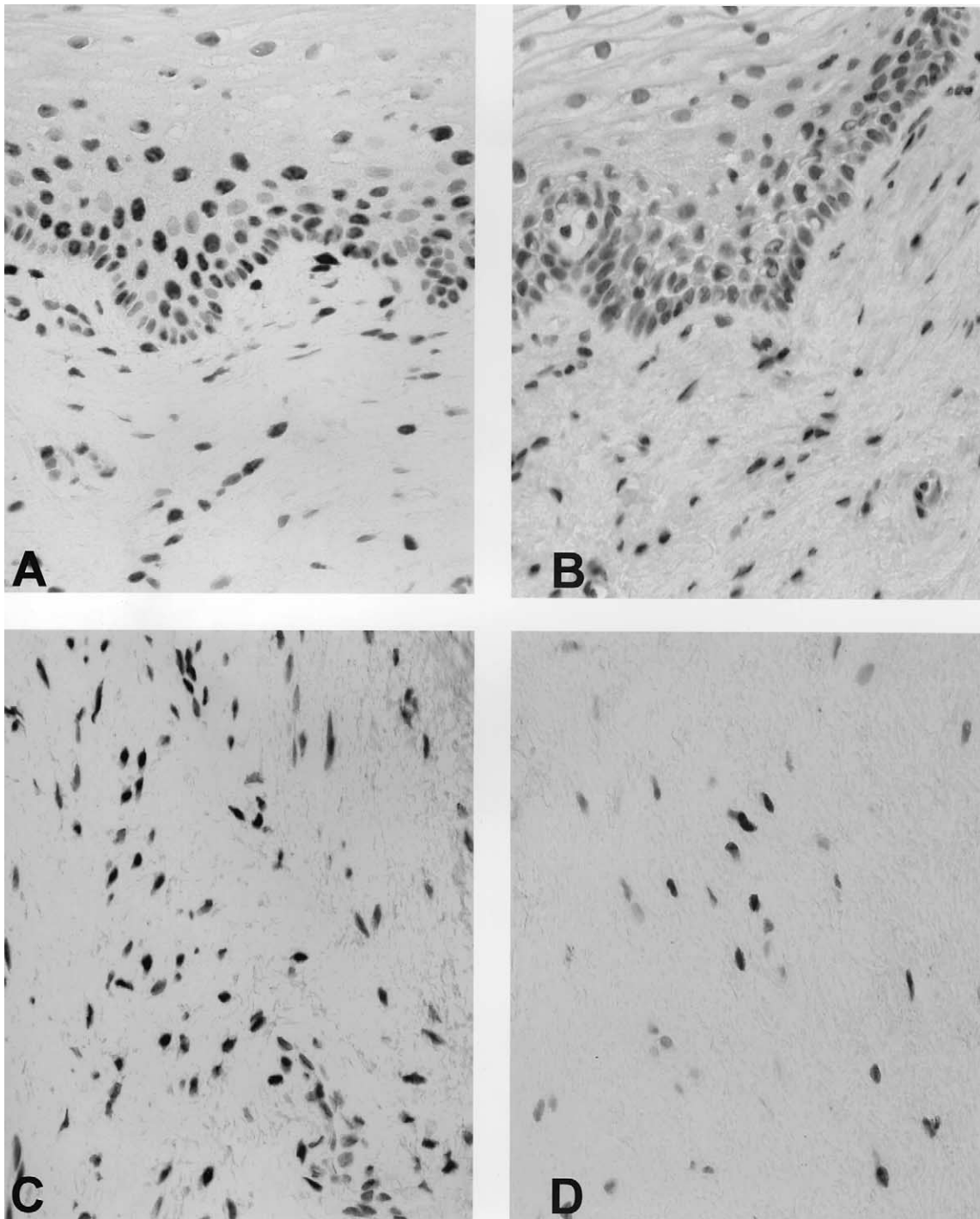
Yes = *P* < .05; No = *P* > .05.

^a Sample size insufficient for making any definite conclusion.

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FIGURE 2

Immunohistochemical detection of vaginal stromal androgen receptors in women with and without hormone therapy (HT). Paraffin-embedded sections were immunostained with an antibody against the androgen receptor. (A), Vaginal stroma and epithelium; (B), negative control; (C), stromal androgen receptor expression from a patient not receiving HT; and (D), from a patient with HT. Magnification, $\times 200$.



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TABLE 2

Androgen in submucosa by vaginal location.

Vaginal location	n ^a	Mean	SEM
Anterior distal	15	1.36	0.36
Anterior proximal	12	3.18	0.40
Posterior distal	11	1.82	0.42
Posterior proximal	12	2.37	0.40

Statistically significant mean differences.

^a n does not add up to 27 since one woman can have data at more than one location. $F = 5.09, P = .001$ *Berman. Vaginal androgen receptors. Fertil Steril 2003.*

Androgen Receptor Density and Menopausal Status

A negative correlation existed between age and androgen receptor density in vaginal mucosa but not submucosa ($r = -0.38; P = .05$, and $r = -0.31, P = .11$, respectively). In premenopausal women, vaginal mucosal androgen receptor density scores were significantly higher (3.84 ± 0.72) than specimens from postmenopausal women on (1.96 ± 0.72) and off (2.20 ± 0.68) estrogen therapy (ET) ($P = .02$ and $.05$, respectively). There was no significant difference between submucosal androgen receptor density scores in premenopausal vs. postmenopausal women. This is consistent with the above-mentioned absence of age-related changes in androgen receptor density in vaginal submucosa.

Postmenopausal women receiving ET (oral and transdermal) had significantly lower vaginal submucosal androgen receptor density scores than postmenopausal women not receiving ET. There were no differences in mucosal androgen receptor density scores between postmenopausal women on and off ET. There were also no differences in vaginal mucosal or submucosal androgen receptor density based on route of HT delivery or presence of P.

These results are summarized in Table 1, where the content of androgen receptors is correlated with demographic and clinical data. Factors associated with a significant difference in mean androgen density score are marked

TABLE 3

Androgen in submucosa by menopausal status.

Menopausal status	n	Mean	SEM
Post-none	6	3.53	0.61
Post-oral	14	1.69	0.25
Post-td	1	1.00	—
Post-vag	1	1.00	—
Pre	5	2.51	0.18

 $F = 4.27, P = .010$.*Berman. Vaginal androgen receptors. Fertil Steril 2003.*

TABLE 4

Androgen in mucosa by prior pelvic surgery.

Prior surgery	n	Mean	SEM
No	6	3.65	0.30
Yes	21	1.99	0.32

 $t = 2.64, P = .014$.*Berman. Vaginal androgen receptors. Fertil Steril 2003.*

by “yes.” Nonsignificant associations are indicated with “no.” Increasing age, distal vaginal location, presence of ET, and history of previous pelvic surgery were all associated with a significantly lower androgen receptor density score. Tables 2 through 5 provides the summary of the statistically significant mean differences. We do not provide the other 14 tables with nonsignificant results.

DISCUSSION

Although T has gained attention as a potential additional component of hormone therapy (HT), little is known about T action on the human vagina. We present the first documented evidence of the expression of aromatase, and 5 α R-I and 5 α R-II, the enzymes necessary for the metabolism of T, in the human vagina. These findings suggest that they play a role in the conversion of T to both dihydrotestosterone and E₂ in the vagina. The presence of aromatase mRNA suggests that some of the effects of T in the vagina are also mediated through conversion to E. In E-depleted women, this residual source of E could be beneficial. Varying levels of aromatase in the vagina may help to explain why postmenopausal women receiving HT present with different degrees of vaginal maturation and atrophy, sometimes requiring the addition of topical E therapy.

This study confirmed that androgen receptors, mediating both T and dihydrotestosterone actions, are present in human vagina, and their density is affected by age, menopausal status, and E replacement. Postmenopausal women receiving oral or transdermal E replacement had lower vaginal androgen receptor densities than those who were not. This suggests that E replacement may down-regulate vaginal andro-

TABLE 5

Androgen in mucosa by age group.

Age group	n	Mean	SEM
31–55	9	3.19	0.64
56–78	18	1.94	0.26

 $t = 2.16, P = .040$. %Spearman correlation (r_s) of androgen in mucosa with age: $r_s = -0.38, P = .05$.*Berman. Vaginal androgen receptors. Fertil Steril 2003.*

gen receptors. Fewer androgen receptors in vaginal subepithelium of women on HT may result from estrogenic stimulation of sex hormone-binding globulin (SHBG), leading to less free T and, therefore, less production of androgen receptors.

Presence of mRNAs for both 5 α R isoforms in the vagina is consistent with what has been found in other tissues both in men and women (21). This suggests that T metabolism in the vagina may follow a similar pattern to that of the androgen-dependent prostate (22, 23). This is in contrast to tissues such as skin or liver, where the much stronger predominance of isozyme I is closely related to steroid catabolism. Further studies are needed to determine whether the relative level of mRNA expression for both isozymes identified by RT-PCR can also be validated by Northern blot, and whether this is reflected at the protein and enzyme levels. Because little is known regarding the distinctive role of both isozymes in the central nervous system and prostate, in terms of male sexual function and response, it is difficult to speculate on what their respective function may be in the vagina.

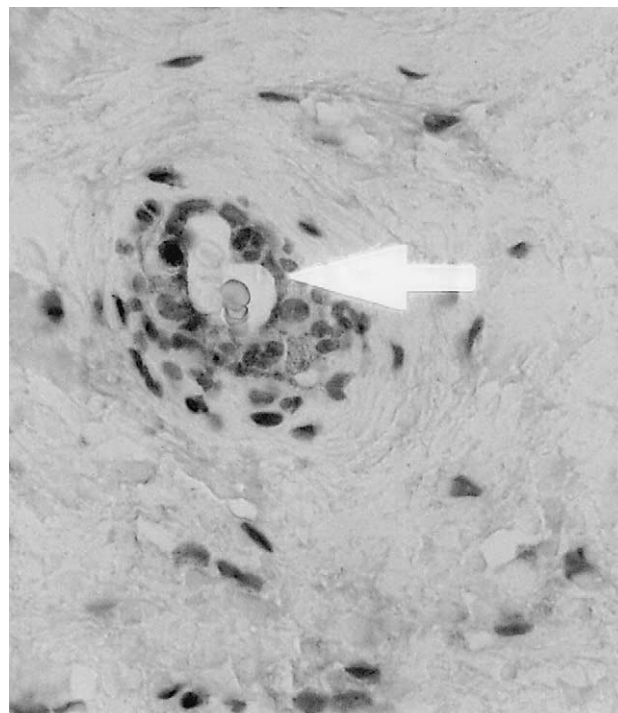
Contrary to previous studies demonstrating no significant differences in density of androgen receptors in pre- vs. postmenopausal women (24), the current study revealed that androgen receptor density is lower in the mucosa of postmenopausal women, irrespective of type or route of HT. It is unclear at this time whether this is due to an age effect or a hormone/menopausal effect, as no determinations have been conducted over an adequate age span. Clearly, reduction of androgen receptors in postmenopausal women combined with the gradual decline in serum androgen levels in women as they age would decrease androgen responsiveness of vaginal tissue even further (10).

In humans, only a few immunohistochemical studies have demonstrated the presence of androgen receptors in female genitalia. Those studies have found more receptors in the vulva compared to the vagina (3). No previous reports have shown differences in androgen receptors based on vaginal location, in terms of proximal vs. distal vagina or mucosal vs. submucosal layers. Due to the different embryologic origins of the proximal and distal vagina, and the different vascular supply and innervation, distinctive functional roles may exist. Although our study and others (3, 4, 25, 26) show that androgen receptors are differentially expressed in the uterus and vagina, estrogen receptors, in contrast, are consistently expressed throughout the vagina, suggesting a uniform role of estrogen in the female reproductive tract (Jolin et al., unpublished observations) (27, 28).

Vaginal mucosa is a mucous-type stratified nonkeratinized squamous cell epithelium that undergoes cyclical changes during the menstrual cycle. Although androgen receptors were present in vaginal mucosa, expression was higher overall in the submucosa. Although age and exogenous ET did not affect submucosal androgen receptor expression, mucosal androgen receptor expression was signif-

FIGURE 3

Immunohistochemical detection of androgen receptors in vascular endothelial smooth muscle of the vagina; *arrow*: androgen receptor staining in vascular endothelial cell.



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icantly diminished in older women and postmenopausal women receiving ET. This suggests that the vaginal epithelium may be more sensitive to fluctuations in E than the submucosa, and may respond to topical androgen therapy.

The submucosal portion of the vaginal wall is known to be highly infiltrated with smooth muscle and has an extensive tree of blood vessels. Previous studies have shown that T replacement to ovariectomized rabbits increases genital blood flow (29). Studies in humans have also revealed a positive effect of androgens on vaginal blood flow (30, 31). In this study, the presence of androgen receptors in submucosal vascular endothelial cells suggests that T may also affect vasculature of human vagina (Fig. 3). In men, androgen receptors are present in endothelial and smooth muscle cells of blood vessels in the prostate, which has been correlated by a stimulating effect of T on the vasculature in the prostate (4). A similar process may be occurring in the vagina. Similar to E, T may also have trophic effects on vaginal smooth muscle, stroma, and vascular endothelium, preventing apoptosis and fibrosis.

At this point it is difficult to understand the physiological significance of the relative abundance of androgen receptors in the proximal vagina. This region of the organ is involved

in sperm survival and capacitance as a result of the alkaline vaginal and cervical secretions. The proximal portion of the vagina also dilates, allowing for pooling of sperm at the level of the cervix. Although no previous studies have evaluated the potential role of androgen responsiveness in these processes, animal studies have demonstrated that T increases vaginal smooth muscle relaxation, above and beyond the effects of E (29, 32).

In conclusion, these data support a potential physiologic role for T in the vagina. mRNA expression of the essential enzymes for T metabolism, aromatase and 5 α R-I and 5 α R-II occurs in the vagina, although further studies are needed to characterize the respective proteins and their specific enzyme activity. The fact that there are regional and cell-type specific (mucosa vs. submucosa) differences in androgen receptor distribution in the vagina and that androgen receptor density is affected by menopausal status suggests that T may play a specific role in the vagina and the sexual responses associated with each region.

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