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Androgen Imbalance in Premenopausal Women with Benign Breast Disease and Breast Cancer

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Objective: The alteration of steroid hormonal status in premenopausal breast disease (benign and malignant) were investigated by comparing the urinary profile of androgens and corticoids.
Methods: The urinary concentrations of 25 androgens and corticoids were quantitatively determined by a gas chromatography-mass spectrometry system in patients with benign breast disease (35 cases, 20–54 years), breast cancer (34, 27–54), and healthy controls of similar age (25, 22–51).
Results: In premenopausal patients with breast cancer, a significantly lower rate of excretion of 11-deoxy-17-ketosteroids and their metabolites was found in comparison with normal females. These levels were also inversely associated with benign breast disease. No significant differences were found between the three groups for the concentration of 11-oxy-17-ketosteroids, 17-hydroxy-corticoids and their metabolites. The urinary ratio of adrenal androgen metabolites to cortisol metabolites [(11-DOKS & M)/11-OKS] declined in the order of normal female control (4.04 ± 0.72; mean $\pm$ SD), breast benign mass (2.29 ± 0.42) and breast cancer (0.94 ± 0.27).
Conclusion: Our data suggest that the hormonal imbalance of androgen deficiency and/or corticoid sufficiency is closely associated with the benign and malignant conditions of premenopausal breast disease and the ratio of (11-DOKS & M)/11-OKS may be an effective discriminant factor of these groups. Copyright © 1999 The Canadian Society of Clinical Chemists

KEY WORDS: breast cancer; benign breast disease; androgens; corticoids; gas chromatography-mass spectrometry.

Introduction

It has been suggested that hormones such as prolactin, progesterone, estrogens, and androgens are in some way implicated in the development and/or growth of normal and neoplastic mammary tissue.

In particular, estrogens are known to be the primary stimulant for breast cell proliferation. It is generally believed that an alteration in estrogen metabolism is closely related to breast cancer risk (1–3). The results of our previous study (4) also support the hypothesis that elevated 16α-hydroxylation and lowered 2-hydroxylation of estrogen metabolism are associated with breast cancer.

Regarding androgens, it has been proposed that androgens are not involved in tumor initiation but control tumor growth rates after the malignant transformation has occurred (5). Swain *et al.* (6) reported that estrogenic and progestational stimuli to the breast are modified by androgen secretion. Recently, androgen receptors (AR) have been analyzed to clarify their clinical significance in breast carcinomas, along with estrogen (ER) and progesterone receptor (PR), but their role remains controversial (7,8).

The most convincing endocrine abnormality in patients with breast cancer has been found to be a subnormal excretion of 11-deoxy-17-ketosteroids in premenopausal women (9–11). It was also suggested that benign and malignant breast disease may be affected by a change in the whole hormonal environment (6,12). Despite many observations, the explanations are often incompatible with each other, and no systematic principle is established concerning the cause of these neoplasia.

The aim of this study is to investigate the role of androgens in benign breast disease and breast cancer in woman before the menopause, as an extension of our previous work on the urinary estrogen profile in breast cancer. The urinary concentrations of 25 androgens and corticoids were quantified using the highly sensitive gas chromatography-mass spectrometry (GC-MS) system. These profiles were compared between patients with benign breast disease, breast cancer and normal female subjects. To find a change of steroid hormonal status in the benign and malignant breast conditions, the concentration ratio of (11-DOKS & M)/11-OKS was determined.

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Methods

Materials

Androgen standards were purchased from Sigma (St. Louis, MO, USA). All solvents were of analytical grade and were used without additional purification. Serdolit AD-2 resin (particle size: 0.1-0.2 mm) was purchased from Serva (D-69115 Heidelberg, Carl-Benz-Str.7, Germany). β-Glucuronidase/arylsulfatase from *Helix Pomatia* was purchased from Boehringer Mannheim (Germany): β-glucuronidase activity was 5.5 U/mL (at 39° C) and arylsulfatase activity was 2.6 U/mL (at 38° C). Deionized water was distilled before use. Silylating reagents, MSHFB (*N*-methyl-*N*-trimethylsilylheptafluorobutyramide) was purchased from Macherey-Nagel (D-5160 Düren, Germany). TMCS (trimethylsilylchloride) and TMSIm (*N*-trimethylsilylimidazole) were purchased from Sigma. Diethyl ether was of a high purity "HPLC solvent" grade and distilled before use.

Subjects and sample collection

Subjects included women with a newly diagnosis of invasive breast cancer ($n = 34$), benign breast disease ($n = 35$), and women with no evidence of benign or malignant breast disease as normal controls ($n = 25$). All cases and controls in this study underwent the same diagnostic procedures, *i.e.*, breast physical examination, mammography, and ultrasonography, in the same facilities. As for patients characteristics, cases and controls were similar in terms of age (mean age of malignant cases; 40.6 ± 6.99 years, benign cases; 38.4 ± 9.71, and 39.6 ± 7.21 years for controls) and demographics. The breast cancer patients groups received no irradiation or hormonal treatment. Early morning urine samples were obtained. The collected urine samples were stored at −20° C without preservative until analyzed. Creatinine was measured by a Jaffé method.

Gas chromatography-mass spectrometry

The Hewlett-Packard GC-MS system (from Hewlett-Packard Co., Palo Alto, CA, USA) consisted of a Model 5890A gas chromatograph, a Model 5970B mass-selective detector, and a HP G1701AA MSD ChemStation. The GC column was a 17 m H 0.2 mm (internal diameter) fused silica capillary, coated with methyl siloxane (film thickness: 0.11 Fm). The carrier gas (helium) flow rate was 0.85 mL/min, and the split ratio was 1:13. The GC temperature program was as follows: the initial temperature (180° C) was programmed at 4° C/min to 300° C and maintained for 2 min. The injector temperature was 300° C, the transfer line was 300° C and the ion source was 200° C. The mass spectrometer was operated at 70 eV in the electron-impact (EI) mode. Selected ion monitoring (SIM) mode was used for quantifying 25 androgen metabolites. The dwell time for each ion was set at 50 msec.

Extraction of androgens and corticoids

A preconditioned Serdolit AD-2 resin was poured into a Pasteur pipette (0.5 cm I.D.) to 3 cm. Three mL of urine and internal standard (cholesteryl isobutyrate, 0.2 μg) were applied to the column. After washing the column with 3 mL of water, the androgens and corticoids were eluted three times with 1 mL of methanol. The eluant was evaporated to dryness in a rotary evaporator. To carry out enzyme hydrolysis, the residue was then dissolved in 1 mL of acetate buffer (0.2 N, pH 5.0) containing 50 μL of β-glucuronidase/arylsulfatase (from *Helix Pomatia*). The sample was incubated overnight at 37° C or for 1 h at 55° C. After the hydrolysis, 100 mg of potassium carbonate was added, and the pH was adjusted to 9.0. The mixture was extracted with 5 mL of diethyl ether, and the organic layer was transferred to another tube for vacuum evaporation. The residue was dried in a vacuum desiccator over P_2O_5-KOH.

The residue was dissolved in 50 μL of the reagent mixture (MSHFB/TMCS/TMSIm, 2:2:1 volume ratio) and heated for 10 min at 60° C. After heating, 2 μL aliquots were injected into the GC column by an autosampler.

Assay

The following 25 androgens and corticoids were measured: androsterone (An), etiocholanolone (Et), dehydroepiandrosterone (DHEA), 4-androstenedione (Δ^4-Dione), 5-androstenediol (Δ^5-Diol), testosterone (Te), dihydrotestosterone (DHT), 16α-hydroxy DHEA (16α-OH DHEA), 5-androstene-3α,16β,17β-triol (Δ^5-AT), 11-keto An, 11-keto Et, 11β-OH An, 11β-OH Et, tetrahydrodeoxy-corticosterone (THDOC), tetrahydro-11-deoxycortisol (THS), tetrahydro-11-dehydrocorticosterone (THA), tetrahydrocortisone (THE), 5β-tetrahydrocortisol (THF), 5α-tetrahydrocortisol (5α-THF), α-cortolone, 5β-tetrahydrocorticosterone (THB), β-cortolone, β-cortol, α-cortol, and 5α-tetrahydrocorticosterone (5α-THB). All values were corrected for concentration of urinary creatinine.

All urine samples were analyzed in separate batches for the two groups within 1-month period together with one duplicate quality-control sample for each batch. The quality-control samples used were pooled urine samples from normal individuals.

The recovery range of the androgen and corticoid extraction method was 72.33–94.54%. It was found to be reproducible and quantitative. The CVs of intraday analysis was 1.43–10.86% and that of interday analysis was 0.96–9.98% (13).

Statistical analysis

All directly measured hormone variables were normally distributed, and the statistical significance

TABLE 1
Concentration of Urinary Endogenous Steroids in Normal Female Subjects and Patients with Breast Benign Mass and Breast Cancer

Endogenous steroids	Normal Female (n = 25)		Benign Breast Disease (n = 35)		(μmol/g of creatinine) Breast Cancer (n = 34)	
	Mean	Range	Mean	Range	Mean	Range
11-Deoxy-17-ketosteroids and their metabolites						
Androsterone [An]	9.26	5.13–17.45	7.74	2.43–13.31	3.70	0.77–10.63
Etiocholanolone [Et]	10.39	4.11–16.45	8.05	1.61–14.04	3.47	0.22–7.31
Dehydroepiandrosterone [DHEA]	1.77	0.34–4.32	0.91	0.13–2.84	0.59	0.10–1.46
4-Androstenedione [Δ^4-Dione]	0.91	0.12–2.02	0.43	0.08–1.60	0.27	0.03–1.10
5-Androstenediol [Δ^5-Diol]	1.01	0.40–3.17	0.45	0.20–2.81	0.25	0.16–1.24
Testosterone [Te]	0.43	0.08–1.43	0.27	0.03–1.01	0.13	nd–0.54
Dihydrotestosterone [DHT]	1.03	0.18–2.87	0.57	0.06–1.28	0.13	0.05–0.62
16α-Hydroxy DHEA [16α-OH DHEA]	3.68	1.00–6.56	2.06	0.29–5.12	0.26	0.07–2.21
5-Androstene-3α,16β,17β-triol [Δ^5-AT]	2.67	1.38–3.14	1.57	0.27–2.73	0.62	0.15–2.03
11-Oxy-17-ketosteroids						
11-Keto An	1.08	0.14–3.14	1.45	0.11–3.54	1.08	0.11–3.33
11-Keto Et	1.03	0.42–3.58	1.56	0.37–2.93	0.85	0.19–2.90
11β-OH An	3.97	1.29–8.52	3.31	1.19–6.07	2.68	0.77–7.53
11β-OH Et	1.78	0.24–2.58	1.96	0.18–3.10	1.85	0.17–2.85
17-Hydroxycorticosteroids and their metabolites						
Tetrahydrodeoxycorticosterone [THDOC]	0.09	0.03–0.31	0.06	0.02–0.20	0.05	nd–0.58
Tetrahydro-11-deoxycortisol [THS]	0.49	0.15–1.48	0.40	0.20–0.90	0.33	0.05–0.95
Tetrahydro-11-dehydrocorticosterone [THA]	0.56	0.08–1.59	0.64	0.10–1.24	0.47	0.09–0.96
Tetrahydrocortisone [THE]	13.36	7.97–25.22	13.35	7.61–32.74	12.53	5.30–23.87
5β-Tetrahydrocortisol [THF]	7.32	3.84–15.55	5.21	1.38–9.23	6.56	1.61–17.59
5α-Tetrahydrocortisol[5α-THF]	5.02	1.48–12.76	6.37	1.89–14.76	7.18	1.27–24.00
α-Cortolone	6.00	1.70–9.26	3.35	0.65–7.00	5.09	1.01–11.96
5β-Tetrahydrocorticosterone [THB]	1.17	0.61–3.80	1.14	0.35–3.61	1.12	0.39–4.21
β-Cortolone	1.57	0.99–2.60	1.19	0.36–3.03	1.19	0.34–3.59
β-Cortol	1.48	0.67–2.56	1.78	0.67–3.75	1.21	0.50–2.59
α-Cortol	1.83	0.87–3.36	1.09	0.42–2.53	1.53	0.40–5.00
5α-Tetrahydrocorticosterone [5α-THB]	1.17	0.37–2.00	1.12	0.35–2.34	1.22	0.36–3.66

nd = not detected.

of the difference in these variables between cases and controls were evaluated using t-test for two independent means or, when appropriate, by the paired t-test. Distribution of (11-DOKS & M)/11-OKS also appears to be normal and statistical analysis for this ratio of significance was conducted by the t-test. As the value of significance, $p < 0.05$ was accepted.

Results

Because treatment for breast cancer could alter the hormonal profiles, we compared the androgen and corticoid profiles in patients who had received no treatment prior to giving urine with age-matched normal female subjects. The excretion of urinary 11-deoxy-17-ketosteroids, 11-oxy-17-ketosteroids, 17-hydroxycorticosteroids, and their metabolites were measured using gas chromatography-mass spectrometry. Table 1 summarizes the concentration levels (mean and range) of these androgen and corticoid metabolites excreted in the urine of 34 patients with breast cancer, 35 patients with benign breast disease, and 25 normal female subjects. The urinary levels of 11-deoxy-17-ketosteroids (An, Et, and DHEA) and their metabolites (Δ^4-Dione, Δ^5-Diol, Te, DHT, 16α-OH DHEA and Δ^5-AT) were significantly lower ($p < 0.05$) in the patients with breast cancer than those found in normal female subjects. These levels were also lower in the patients with benign breast disease as compared to normal controls, but statistical significance was not achieved.

There were no differences between the three groups in the excretion of 11-oxy-17-ketosteroids (11-keto An, 11-keto Et, 11β-OH An and 11β-OH Et) and 17-hydroxycorticosteroids (THS, THE, THF, 5α-THF, α-cortolone, β-cortolone, β-cortol, and α-cortol) and their metabolites (THDOC, THA, THB, and 5α-THB).

The ratio of the sum of 11-deoxy-17-ketosteroids and their metabolites to the sum of 11-oxy-17-ketosteroids [(11-DOKS & M)/11-OKS] was determined in normal female subjects and in patients with breast diseases and shown in Table 2. In normal controls, the highest mean value (4.04 ± 0.72; mean $\pm$ SD) of (11-DOKS & M)/11-OKS was observed. This ratio was found to be decreased in the patients with benign breast diseases (2.29 ± 0.42)

TABLE 2
Total 11-Deoxy-17-Ketosteroids and Their Metabolites and 11-Oxy-17-Ketosteroids and Ratio of (11-DOKS & M)/11-OKS in Urine of Normal Female Subjects and Patients with Benign Breast Diseases and Breast Cancer

	Normal Female		Benign Breast Disease		Breast Cancer	
	Mean	SD	Mean	SD	Mean	SD
Total value						
Sum of 11-deoxy-17-ketosteroids and their metabolites [11-DOKS & M]	32.16	7.65	20.11	5.23	9.33	3.12
Sum of 11-oxy-17-ketosteroids [11-OKS]	8.02	2.26	8.69	2.10	8.54	2.25
Ratio						
(11-DOKS & M)/11-OKS	4.04	0.72	2.29	0.42	0.94	0.27

and breast cancer (0.94 ± 0.26). Patients with benign breast disease had significantly lower mean values than normal controls ($p < 0.01$). Also, the mean value of (11-DOKS & M)/11-OKS was significantly lower in patients with breast cancer as compared to the benign cases ($p < 0.01$). Our data on the urinary ratio of (11-DOKS & M)/11-OKS are illustrated in Figure 1 for the three groups.

Discussion

The alteration of the endogenous hormonal environment is known to affect the breast cancer. Numerous studies on the endogenous hormonal profiles of breast cancer patients have been performed. With regard to androgens, it was suggested that they have a major part to play in the control of tumor growth (5). Two hypotheses are available for the relationship between androgens and breast cancer risk: the hypothesis of increased risk with adrenal androgen deficiency, and of ovarian dysfunction (luteal inadequacy and excessive ovarian androgen secretion) (14). Our results for the androgen estimations provides additional evidence for the former hypothesis.

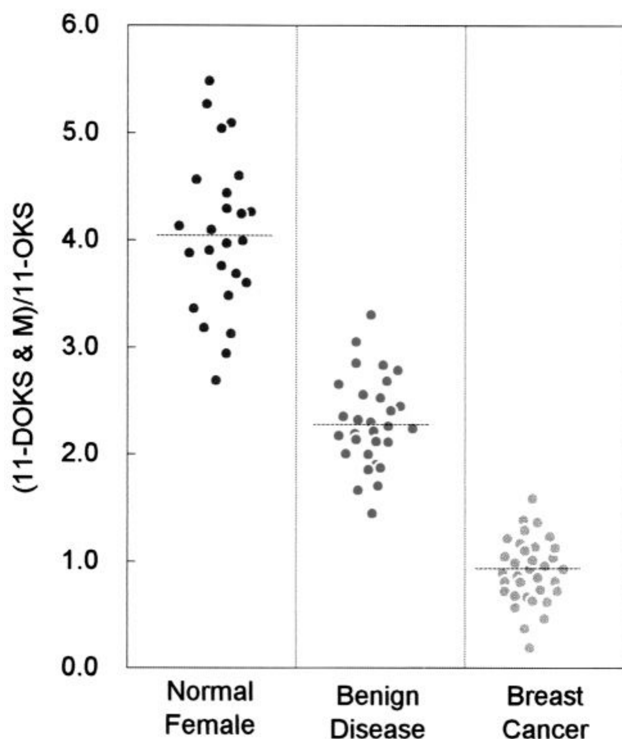

Figure 1 — Urinary ratio of (11-DOKS & M)/11-OKS in normal premenopausal women and in patients with benign and malignant breast disease. The median value in each group is indicated by a broken line (- - - -).

In this study, the 11-deoxy-17-ketosteroids, the 11-oxy-17-ketosteroids, the 17-hydroxycorticosteroids and their metabolites were analyzed in the urine of premenopausal patients with benign breast disease, breast cancer and normal females. It was found that not only the urinary excreted amounts of the individual 11-deoxy-17-ketosteroids (An, Et and DHEA) but also those of their metabolites (Δ^4-Dione, Δ^5-Diol, Te, DHT, 16α-OH DHEA and Δ^5-AT) were significantly lower in patients with breast cancer than in the normal female controls ($p < 0.05$, statistical data not shown). It was also observed that there were no significant changes in the concentration (mean and range) of the 11-oxy-17-ketosteroids, the 17-hydroxycorticosteroids and their metabolites. Our results support the previous studies which reported subnormal urinary excretion of adrenal androgen metabolites in premenopausal women with breast cancer (15,16) prior to mastectomy (17). Allen *et al.* (15) and Kumaoka *et al.* (18) observed that urinary 17-hydroxycorticosteroids were normal in most patients with advanced breast cancer. This is also in accordance with the previous suggestion of Bulbrook *et al.* (5) in which the androgens generally inhibit tumor growth rates.

Although benign breast mass is known to have no clinical relevance to breast cancer, there are some evidences that women with benign breast disease have a greater risk of subsequent breast cancer (19–21). It is thought that, therefore, a difference of hormonal status, if any, in benign and malignant breast disease might provide further insight into the role of androgens in breast cancer as well as the etiology of the benign conditions. By this reasoning, the urinary levels of androgens and corticoids were also determined for the patients with benign breast disease in this investigation. A discernible reduction

was also observed in the excretion of 11-deoxy-17-ketosteroids and their metabolites in premenopausal benign breast disease in comparison with normal female controls but not statistically significant. The values of the mean and range in these women were distributed between that of normal and breast cancer cases.

To investigate the possible involvement of steroid-hormone environment (androgens-corticoids balance) in benign and malignant status of breast diseases, the ratio of 11-DOKS & M (the sum of 11-deoxy-17-ketosteroids and their metabolites levels) to 11-OKS (the sum of 11-oxy-17-ketosteroids levels) was evaluated in the urine of normal subjects and patients with benign breast disease and breast cancer. The 11-DOKS & M are derived from adrenal androgens and the 11-OKS are cortisol metabolites of low androgenicity. As seen in Table 2, this ratio declined in the order of normal female, benign breast disease, and breast cancer. That is, our current results indicate that the hormone balance is shift from the androgen dominance to the corticoid dominance according to progress the malignant transformation.

Between the three groups, differences of mean and range value were obtained statistically ($p < 0.01$). The distribution of this ratio for normal female, breast benign disease and breast cancer is described in Figure 1, which demonstrates distinctly that patients with breast cancer could be differentiated from patients with benign breast disease as well as normal females in terms of the ratio of (11-DOKS & M)/11-OKS. From this observation about the ratio of (11-DOKS & M)/11-OKS, the possibility was considered that a change in the balance of adrenal androgens and corticoids is closely associated with the benign and malignant status of premenopausal breast disease and this imbalance also lead to an abnormal stimulus to breast tissue by an alteration of estrogen metabolism (16α- or 2-hydroxylation of estrone) in premenopausal benign breast disease and breast cancer cases.

In conclusion, the present investigation with androgen and corticoid profiles provides further evidence that an important relationship exists between decreased urinary levels of adrenal androgens (11-deoxy-17-ketosteroids and their metabolites) and breast cancer in premenopausal women. From the variation of the ratio of (11-DOKS & M)/11-OKS, it is suggested that the hormonal imbalance of androgen deficiency and/or corticoid sufficiency is closely associated with the benign and malignant conditions of premenopausal breast disease and this ratio may be an effective discriminant factor of these groups.

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