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Urinary Profiles of the Endogenous Steroids in Pre-Menopausal Women with Uterine Leiomyoma

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자궁근종 성장에 있어서 내인성 성호르몬의 역할

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목 적: 본 연구의 목적은 내인성 성호르몬이 자궁근종의 성장에 미치는 영향을 알아보고자 연구를 시행하였다.

연구대상 및 방법: 폐경전 자궁근종 환자 27명과 같은 연령대의 정상여성 25명을 대상으로 하였다. 모든 대상군의 여성에서 24시간 소변을 모아서 소변내 estrogen, androgen의 대사체들을 GC-MS를 이용하여 측정하였으며 두 군에서의 차이를 비교분석 하였다.

결 과: 소변내 17ß-estradiol, 5-androstene-3ß, 16ß, 17ß-triol, 11-keto-ethiocholanolone, 11ß-hydroxy-androsterone, THS, THA, THE, a-cortolone, a-cortol 및 ß-cortol가 환자군에서 의의있게 증가하였으며 17ß-estradiol/estrone 및 11ß-hydroxy-ethiocholanolone/11ß-hydroxy-androsterone도 환자군에서 의의있게 증가하였다.

결 론: 자궁근종의 성장은 요중 estrogen과 androgen의 농도와 밀접한 관련이 있으며 이는 환자의 스테로이드 호르몬 대사 감소에 기인한 것으로 사료된다.

중심단어: 자궁근종, 스테로이드, 에스트로젠, 안드로젠, 요중농도

Uterine leiomyoma is the most common solid pelvic tumor, occurring in 20~30% of women who are over 30 years of age[1] and it accompanies with the symptoms such as uterine bleeding, dysmenorrhea, pain and the pressure on the urinary tract lead to blockage of the urinary tract. It sometimes becomes a factor in sterility.[2] Leiomyoma is a benign neoplasm that arises from uterine smooth muscle. It is hypothesized that leiomyoma originates from the somatic mutations in myometrial cells, resulting in progressive loss of growth-regulation.[3,4]

Ovarian hormones are believed to stimulate the growth of leiomyoma because there is an increased incidence of leiomyoma after menarche and these tumors enlarge during pregnancy and regress after menopause. The growth of leiomyoma is variable among women with regular menstruation cycles and even among myoma nodules in the same uterus. One possible reason for this variation is that

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ovarian hormones, especially estrogen, stimulate individual myoma nodules by varying degree.[5]

Therefore, therapeutic attempt based on overcome the state of hyperestrogenism have been tried. Treatment with competitive inhibitors of estrogen receptors (ER)[6,7] or gonadotropin-releasing hormone agonist[2,8,9] has been studied for those reasons. It was found that mean ER content was significantly greater in leiomyoma than in myometrium.[10~14] And ER content of the fibroid was reported to significantly correlate with the myoma-shrinkage.[15] But there were no consistent results as the concentration of estrogen in uterine leiomyoma.[16,17]

In this study, we determined the concentrations of urinary steroids, including estrogens as well as androgens, which are closely related to the estrogen biosynthesis, in premenopausal women with leiomyoma using Gas Chromatography-Mass Spectrometry (GC-MS). The urinary levels of the same endogenous steroids in age-matched healthy premenopausal women were also estimated by comparing urinary steroid levels between the two groups.

From these results, we studied the effect of endogenous steroids and the metabolic changes in the leiomyoma, and we especially observed the difference in the estrogen level between the two groups to predict the role of estrogens in the prevention of and the therapy for leiomyoma.

MATERIALS AND METHODS

1. Materials

Androgen and estrogen standards were purchased from Sigma (St. Louis, Mo, USA). d_2-17ß-estradiol used as an internal standard for the estrogen profile was purchased from MSD Isotope (Montreal, Canada). All reagents 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 (Heidelberg, Germany). ß-glucuronidase/arylsulfatase from *Helix Pomatia* was purchased from Boeringer Mannheim (Germany). ß-glucuronidase activity was 5.5 U/ml (at 39℃) and aryl sulfatase activity was 2.6 U/ml (at 38℃). Silylating reagents, MSHFB (N-methyl-N-trimethylsilyheptafluorobutyramide) was purchased from Machery-Nagel (Duren, Germany), and MSTFA (N-methyl-N-trimethylsilyltrifluoroacetamide), TMCS (trimethylsilylchloride), and TMSIm (N-trimethylsilylimidazole) was purchased from Sigma (St. Louis, MO, USA). Ethylacetate and ether were of a high purity "HPLC solvent" grade, and ether was distilled before use.

2. Subjects and sample collection

The subjects consisted of pre-menopausal women with uterine leiomyoma (n=27, patients) and healthy, pre-menopausal women with no evidence of myoma (n=25, controls). All patients underwent pelvic and ultrasound exams, and the presence of the myoma was confirmed by surgical pathology. All subjects were in the late luteal phase of menstrual cycle. The ages of the women were 43.1±5.6 and 39.6±7.2 years old, the weights 61.4±7.3 and 54.9±8.1 kg, and the heights 159.3±8.3 and 155.1±4.8 cm in the patients and in controls, respectively. They were all Korean and nonsmokers. The urine samples were colleted for 24 hrs, and the urine samples were stored at -20℃ until analysis. The creatinine value for each sample was measured by the Jaffé method.

3. Gas Chromatography-Mass Spectrometry

The Hewlett-Packard GC-MS system consisted of a gas chromatography (HP 5972) and mass spectrometry (HP 5989B mass engine). The GC column used for the analysis of estrogens was a fused-silica capillary coated with cross-linked 5% phenylmethyl siloxane (length: 25 m; inner diameter: 0.2 mm; film thickness: 0.33 μm). For the

analysis of androgens, a fused silica capillary, coated with phenylmethylsiloxane (length: 17 m; inner diameter: 0.2 mm; film thickness: 0.11 μm) was used. Helium was used as a carrier gas (the flow rate was 0.85 ml/min), and the split ratio was 1:10. The GC temperature program was as follows. In the case of estrogens, the initial temperature of 180°C was increased to 260°C at a rate of 20°C /min and maintained for 6 min, then the temperature was increased to 275°C at a rate of 2°C/min and maintained for 8 min. Finally, it was increased to 300°C at a rate of 15°C/min and maintained for 10 min. And for androgens, 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. The selected ion-monitoring (SIM) mode was used for quantifying the 20 estrogens and the 21 androgens. The dwell time for each ion was set at 50 msec.

4. Extraction of estrogens and androgens

A preconditioned Serdolit AD-2 resin was poured into a pasteur pipette (inner diameter-0.5 cm) to 3 cm. The urine sample (3 ml) and internal standard (d_2-17ß-estradiol, 1.5 μg for estrogens and methyl testosterone, 5 μg for androgens) were applied to the column. After the column was washed with 3 ml of water, the free and conjugated endogenous steroids were eluted three times with 1 ml of methanol. The eluent was evaporated to dryness in a rotary evaporator. Enzyme hydrolysis was performed using ß-glucuronidase/arylsulfatase (from *Helix Pomatia*) with 1 ml of acetate buffer (0.2 N, pH 5.0) at 55°C for 3 hrs. To prevent the oxidation of catechol estrogens, ascorbic acid (1 mg/ml) was added at the hydrolysis of estrogens. After hydrolysis, potassium carbonate was added, and the pH was adjusted to 9.0. The mixture was extracted with 5 ml of ethyl acetate for estrogens and 5 ml of ether for androgens, respectively. The organic layer was transferred to another tube and dried using a vacuum evaporator. The residue was dried in a vacuum desiccator over P_2O_5/KOH in order to complete removal of moisture. The residue was derivatized with the derivatizing reagents to improve the detection in GC-MSD. The derivatization was performed by using the mixture of MSTFA/TMCS (100:1, volume ratio) at 60°C for 30 min for estrogens and MSHFB/TMCS/TMSIm (2:2:1, volume ratio) at 60°C for 10 min for androgens. After cooling, 2 μl of the aliquots was injected into the GC column by an auto sampler.

5. Assay

The following 18 estrogens (estrone [E1], 17ß-estradiol [E2], 2-hydroxyestrone [2-OH E1], 2-hydroxyestradiol, 2-methoxyestrone [2-Meo E1], 17a-estradiol, 6-dehydroestrone, 6a-hydroxyestradiol, 4-methoxyestradiol, estriol [E3], 16-epiestriol [16-Epi E3], 16, 17-epiestriol, 16a-hydroxyestrone [16a-OH E1], 17-epiestriol, 6-ketoestriol, 2-methoxyestriol, 6-hydroxyestriol, and 16-ketoestradiol [16-Keto E2]) and 24 androgens (androgen [An], etiocholanolone [Et], dehydroepiandrosterone [DHEA], 4-androstenedione [?^{4}-dione], testosterone [Te], dihydrotestosterone [DHT], 5-androstenediol [?^{5}-diol], 11ß-hydroxy An [11ß-OH An], 11ß-hydroxy Et [11ß-OH Et], 11-keto An, 11-keto Et, tetrahydrodeoxycorticosterone [THDOC], 16a-hydroxy DHEA [16a-OH DHEA], 5-androstene-3a, 16ß, 17ß-triol [5-AT], tetrahydro-11-deoxycortisol [THS], tetrahydrocortisone [THE], 5a-tetrahydrocortisol [5a-THF], 5ß-tetrahydrocortisol [THF], a-cortolone, ß-cortolone, a-cortol, ß-cortol, 5a-tetrahydrocorticosterone [5a-THB] and 5ß-tetrahydrocorticosterone [THB]) were determined. All values were corrected by concentration of urinary creatinine.

All urine samples were analyzed in separate

batches for the two groups within a 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 extraction of androgens was 72.3~94.5%. The CVs (coefficient of variances) of intraday analysis was 1.42~10.86% and that of interday analysis was 0.96~9.98%.[18] In regard to the extraction for estrogens, the recovery range was 81.0~97.8% and the CVs of intraassay and interassay were 1.05~10.24% and 0.24~10.52%, respectively.[19] This result approved that the analytical methods used in this study were reliable for the determination of the urinary steroids.

6. Statistical analysis

Statistical analysis for the level of significance was conducted by the t-test for two independent means using the SAS system for windows V9.00 (SPSS Institute Inc. IL, Chicago, USA).

RESULTS

1. Profiles of estrogens

Estrone and 17ß-estradiol were detected in all subjects (both of patients with leiomyoma and controls). Table 1 shows the concentrations of estrone and 17ß-estradiol. Trace amounts of hydroxylated and methoxylated metabolites, such as estriol and 2-methoxyestrone, were detected in the

Table 1. Concentrations (nmole/g creatinine, mean ± SD) of urinary estrogens in normal female subjects (controls, n=25) and patients (patients, n=27) with uterine leiomyoma

Compounds	Control	Patient	p value
Estrone	22.8±8.81	22.9±10.2	NS
17ß-estradiol	20.8±4.46	29.4±11.1	<0.05

Table 2. Concentrations (µmole/g creatinine, mean ± SD) of urinary androgens in normal female subjects (controls, n=25) and patients (patients, n=27) with uterine leiomyoma

Compounds	Controls	Patients	p value
Androsterone (An)	1.05±0.45	1.26±1.26	NS
Etiocholanolone (Et)	0.96±0.41	1.04±1.08	NS
5-Androstene-3ß, 16ß, 17ß-triol (5-AT)	0.26±0.10	1.06±0.94	p<0.01
11-keto Et	0.32±0.18	0.98±1.38	p<0.05
11ß-hydroxy An	0.69±0.29	1.25±1.11	p<0.05
11ß-hydorxy Et	0.29±0.12	1.22±1.87	p<0.05
Tetrahydro-11-deoxycortisol (THS)	0.27±0.10	0.61±0.63	p<0.05
Tetrahydro-11-dehydrocorticosterone (THA)	0.40±0.13	1.72±1.55	p<0.05
Tetrahydrocortisone (THE)	10.0±5.07	3.19±4.35	p<0.01
5ß-Tetrahydrocortisol (THF)	1.25±0.75	2.16±3.75	NS
a-cortolone	2.16±0.79	3.37±4.05	p<0.05
ß-cortolone	2.14±1.27	0.94±0.71	NS
a-cortol	0.60±0.22	1.11±1.06	p<0.05
ß-cortol	0.12±0.31	1.64±1.92	p<0.01
5a-THB	0.55±0.14		
5a-THF	0.56±0.08		

Table 3. Concentration ratios (mean ±SD) of etiocholanolone to androsterone (Et/An), 11ß-OH etiocholanolone to 11ß-OH androsterone (11ß-OH Et/11ß-OH An) and estradiol to estrone (E2/E1) in normal female subjects (controls, n=25) and patients with leiomyoma (n=27)

Concentration ratio	Controls	Patients	p value
Et/An	1.12±0.34	0.95±0.38	NS
11ß-OH Et/11ß-OH An	0.45±0.35	0.84±0.76	<0.05
E2/E1	0.89±0.78	2.01±1.60	<0.01

urine of controls and patients, but it is impossible to quantify the findings due to the low level, which was below detection limits.

The urinary concentration of estradiol was significantly higher ($p<0.05$) in the patients with leiomyoma than in controls but the concentration of estrone was not significantly different between the two groups. The mean E2/E1 ratio was significantly higher ($p<0.01$) in the patients with leiomyoma than in controls (Table 3).

2. Profile of Androgens

The urinary concentrations of androgens are presented in Table 2. DHEA, ?^{4}-dione, ?^{5}-diol, Te, DHT, 16a-OH DHEA, 11-keto An, THDOC, 5a-THF, THB, and 5a-THB were not detected in the urine of neither controls nor patients. But 5-AT, 11-keto Et, 11ß-OH An, 11ß-OH Et, THS, THA, THE, a-cortolone, a-cortol and ß-cortol were significantly higher in the patients with leiomyoma than controls. The urinary concentration ratio of 11ß-hydroxy-ethiocholanolone/11ß-hydroxy-androsterone increased in the patients with leiomyoma, however, there was no significant difference in the urinary concentration ratio of Et/An between the two groups.

DISCUSSION

Uterine leiomyoma is a tumor closely related the estrogen and it has been noted that the development of leiomyoma depends on the condition of menstruation, for example pre-, postmenopause, and pregnancy. It was also found that estrogen receptors increased in leiomyoma when compared with the myometrium in the patients with leiomyoma,[10~14,17] but some contrary results were reported on the concentration of estrogens.

Otubu JA et al.,[15] Potgieter HC et al.[16] and Wu J et al.[17] found that the concentration of 17ß-estradiol was significantly higher in leiomyoma than normal myometrium. On the other hand, Potgieter HC et al.[16] also reported that the serum estrogen level did not differ significantly between the patients with leiomyoma and normal subjects.

After the GC-MS determination of urinary endogenous steroids in this study, the urinary concentrations of 17ß-estradiol was significantly higher in the patients with leiomyoma than controls.

This finding does not correspond to the result of Potgieter HC et al.[16] considering that the urinary level is a reflection of the plasma level.

It is thought that increase of 17ß-estradiol is closely related to the significant increase of E2/E1 ratio ($p<0.05$) in the patients with leiomyoma (Table 3). Because 17ß-estradiol is converted to estrone through the oxidation by 17ß-hydroxy steroid dehydrogenase, it means that metabolic change in the conversion of 17ß-estradiol to estrone significantly decreased in the patient with leiomyoma. This result agrees with the results of Pollow K et al.[20]

In the urinary levels of androgens, 5-AT, 11-keto Et, 11ß-OH An, 11ß-OH Et, THS, THA, THE, a-cortolone, a-cortol and ß-cortol were significantly higher in the patients with leiomyoma than controls.

Androgens are known to be the precursors of

estrogens in biosynthesis. Actually, androstenedione was metabolized by the human endometrium, myometrium and leiomyomata in vitro,[21] and aromatase inhibitor, 14a-hydroxy-4-androstene-3, 6, 17-trione (new androstenedione derivative), inhibits estrogen biosynthesis in human uterine tumors.[22]

For that reason, urinary concentrations of androgens were determined in this study, and the activity of 5a-reductase was examined by the urinary concentration ratios of Et/An and 11ß-OH Et/11ß-OH An.

Similar to the results of estrogens, the concentration ratio of 11ß-OH Et/11ß-OH An was higher in the patients with leiomyoma than controls. However, there was no significant difference in the urinary concentration ratio of Et/An. This indicates that androgens metabolism also partially decreased in the disease state of leiomyoma.

In conclusion, it is thought that the growth of uterine leiomyoma correlates with the increase of the urinary level of estrogen and androgen, and this seems to be caused by the decrease of steroid metabolism in the patients with leiomyoma.

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