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Altered urinary profiles of polyamines and endogenous steroids in patients with benign cervical disease and cervical cancer

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Abstract

The risk of cancer of the cervix is linked with sexual behavior. Although infectious agents, such as human papillomaviruses (HPVs) are implicated, these alone may be insufficient to induce the disease. We investigated the potential role of estrogen, androgen, and polyamine metabolism as co-factors in the development of cervical cancer. We obtained urine samples from patients with benign cervical disease ($n = 18$) and cervical cancer ($n = 18$) and from age-matched normal female subjects ($n = 25$). For 11 polyamine determination, an improved and sensitive gas-chromatographic with nitrogen/phosphorus-detection (GC/NPD) procedure was used. The urinary levels of 25 androgens and corticoids and 16 estrogens were quantitatively determined by gas chromatography-mass spectrometry-selected ion-monitoring (GC/MS/SIM). In the patients with cervical cancer, the ratio of 16α-hydroxy estrone (16α-OH E1)/2-hydroxy estrone (2-OH E1), putrescine (Put)/N^1-acetylspermidine (N^1-acSpd) and 5β-tetrahydrocortisol (THF)/5α-tetrahydrocortisol (5α-THF) were significantly increased in comparison to the values of the normal controls. These data suggest: (1) an increase of 16α-hydroxylation in estrogen metabolism; (2) the high activity of polyamine oxidase (PAO) in polyamine metabolism; and (3) the low activity of 5α-reductase in androgen metabolism may play a significant role in the development of cervical cancer. Although additional research is necessary, the combination of 16α-OH E1/2-OH E1 and THF/5α-THF may provide a dual marker for the discrimination of benign cervical disease and cervical cancer.

Keywords: Cervical cancer; Polyamines; Estrogens; Androgens; Corticoids; Gas chromatography-mass spectrometry; Gas chromatography-nitrogen-phosphorus detection

1. Introduction

The etiology of cervical cancer is still the subject of debate. Epidemiological studies have demonstrated that one risk of developing cervical cancer is related to sexual behavior. This finding suggests that the infectious agents, such as human papillomavirus (HPV), are involved. In subsequent studies to discover or determine risk factors for the early detection of cancer, it has been shown that the most important risk factor for cervical cancer is infection with HPV [1]. However, despite this association, HPV alone is not sufficient to induce the disease. Most women who are HPV positive show no

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evidence of cervical abnormalities [2] and only a small proportion of women with HPV develop cervical cancer [3]. Some investigators reported that the difference between these incidences was due to other additional factors. Some factors include the following: (a) an alteration in the endocrine metabolism, as well as environmental factors, such as (b) the long-term use of oral contraceptives [4], (c) heavy smoking, and (d) dietary consumption level of vegetables and fruits [5].

Although their physiological role is not clear, polyamines, steroid hormones, and their metabolites have been found to be associated with the development of cervical cancer. Several reports suggest a remarkable enhancement of polyamine contents in the serum, plasma, and urine of cancer patients with respect to normal individuals [6–9]. Polyamines also mediate immunosuppressive effects in vitro through their oxidation products, which may play a role as a cofactor in the development of cervical cancer [6]. Some researchers suggest that altering the estrogen–hydroxylation metabolism may be a risk factor for cervical carcinogenesis [10,11]. The most estrogen-sensitive cells are at the greatest risk for the HPV-related cancers [10] and papillomavirus lesions are exacerbated during pregnancy when estrogen levels are elevated [12]. The major metabolites of estrone by cytochrome P-450 enzymes are those hydroxylated at either the C-2 or C-16α positions [11]. The increased conversion of estradiol to 16α-metabolites (estriol and 16α-hydroxy estrone) is known to be a marker and/or an indicator of cancer in some other estrogen-sensitive cells [9,11]. The metabolites of the alternate pathways, 2-hydroxyestrone and 2-methoxyestrone are devoid of peripheral estrogenic activity [11]. Therefore, the relative activity of the two enzymes determining the direction of estrogen metabolism may be useful markers for cervical cancer.

Steroid hormones interfere with persistent papillomavirus infections, which were shown to be the risk factor for the later development of cervical cancer [13]. It has also been proposed that androgens are not involved in tumor initiation, but control tumor growth rates [14]. In general, the transcriptional activity of the high-risk papillomavirus promoter elements is enhanced by steroid hormones [15]. Glucocorticoids were shown to enhance viral oncogene expression, thereby leading to increased growth either in vitro or in vivo [16]. Vesanen et al. [17] reported that 5α-reductase metabolites, such as 5α-dihydrotestosterone (5α-DHT), may be related to the altered endocrine metabolism of bovine reproductive tissues.

This study was performed to investigate the possible involvement of polyamines and endogenous steroids in the development of cervical carcinoma. The study was undertaken to understand the changes of polyamine, androgen and corticoid, and estrogen profiles in the urine of patients with benign cervical disease and cervical cancer and in normal female subjects. In order to estimate the various enzyme activities and find useful markers for the early determination of cervical cancer, the concentration ratios of precursor metabolites to product metabolites were compared among the three groups.

2. Materials and methods

2.1. Materials

The polyamine standards, 1,3-diaminopropane (1,3-Dap), putrescine (Put), cadaverine (Cad), spermidine (Spd), spermine (Sp), *N*-acetylputrescine (*N*-acPut), *N*-acetylcadaverine (*N*-acCad), N^1-acetylspermidine (N^1-acSpd), N^8-acetylspermidine (N^8-acSpd), and 1,6-diaminohexane (used as an internal standard) were purchased from Sigma (St. Louis, MO, USA). N^1,N^{12}-diacetylspermine was synthesized as previously described [18].

For estrogen analysis, estrone (E1), 17α-estradiol (17α-E2), 17β-estradiol (17β-E2), estriol (E3), 2-methoxyestrone (2-Meo E1), 2-hydroxyestrone (2-OH E1), 4-methoxyestradiol (4-Meo E2), 16α-hydroxyestrone (16α-OH E1), 2-hydroxy-3-methoxyestradiol (2-OH, 3-Meo E2), 2-methoxyestradiol (2-Meo E2), and 16-epiestriol (16-Epi E3) were obtained from Sigma (St. Louis, MO, USA). d_2-Estradiol, used as an internal standard, was purchased from MSD Isotope (Montreal, Canada).

The following androgen and corticoid standards, 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, tetrahydrodeoxycorticosterone (THDOC), tetrahydro-11-deoxycortisol (THS), tetrahydro-11-dehydrocorticosterone (THA), tetrahydrocortisone (THE), 5α-tetrahydrocortisol (THF), 5α-tetrahydrocortisol (5α-THF), α-cortolone, 5β-tetrahydrocorticosterone (THB), β-cortolone, β-cortol, α-cortol, and 5α-tetrahydrocorticosterone (5α-THB), and internal standard, cholesteryl *iso*-butylate, were purchased from Sigma (St. Louis, MO, USA).

Serdolit XAD-2 resin (particle size 0.1–0.2 mm) was purchased from Serva (Heidelberg, Germany). Sep-pak silica cartridge was from Waters (Milford, MA). 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.

Silylating reagent, *N*-methyl-*N*-trimethylsilylheptafluorobutyramide (MSHFB) was obtained from Macherey-Nagel (Duren, Germany). Heptafluorobutyric anhydride (HFBA), *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), trimethylsilylchloride (TMCS) and *N*-trimethylsilylimidazole (TMSIm) were purchased from Sigma (St. Louis, MO, USA).

Other chemicals and solvents were analytical-reagent grade. Diethyl ether, as an extraction solvent, was distilled from calcium hydride powder just before use.

2.2. Subjects and sample collection

The analysis was performed on 61 females, including 36 patients with the following diagnoses: 18 patients with benign cervical disease with no invasion by a periodical inspection of Pap smear and cervicogram, 18 patients with cervical cancer, and 25 normal controls with no evidence of benign cervical disease or cervical cancer. All of the cases and the controls in this study underwent the same diagnostic procedures in the same facilities. Subjects who were pregnant, who were taking hormone medications, who were using oral contraceptives, or who had complications such as heart disease and thyroid disease were excluded. Urine samples from 25 healthy volunteers (ranging in age from 22 to 47 years), from 18 patients with benign cervical disease (ranging in age from 24 to 52 years), and from 18 patients with cervical cancer (ranging in age from 23 to 50 years) were collected in polyethylene bottles. Early-morning urine samples were obtained, and the collected urines were stored at −20 °C until analysis. Creatinine was measured by a Jaffé method.

2.3. Polyamines analysis

For the extraction of urine samples, to a 2 ml aliquot of urine adjusted to pH 1.0, was added 0.5 μg of internal standard (1,6-diaminohexane). The mixture was extracted with 5 ml of ethylacetate, and the organic phase was discarded. To the aqueous layer 2 ml of a borate buffer (pH 9.0) was added, adjusted to pH 9.0 by adding approximately 200 μl of 4 M sodium hydroxide, and then it was vortexed. The supernatant was applied on a 'Sep-Pak' silica cartridge preconditioned with a 0.1 M methanolic hydrochloric acid solution and water. The 'Sep-Pak' column was washed with 3 ml each of *n*-pentane and methanol, and then the free and acetylated polyamines were eluted with 6 ml of a 0.1 M solution of hydrochloric acid in methanol. The eluate was evaporated to dryness in vacuo. Then, the polyamines were derivatized with a mixture of 100 μl each of ethylacetate and heptafluorobutyric anhydride by heating at 80 °C for 1 h. After cooling, the solvent was evaporated at room temperature under a stream of air, and the residue was dissolved in 50 μl of ethylacetate, and 2 μl aliquots were injected onto the GC column using an autosampler.

For gas chromatography with nitrogen-phosphorus detection, a model 5890 gas chromatograph equipped with a model 7673 automated sampler (both from Hewlett-Packard Co., Palo Alto, CA, USA) was used. The GC column was a 25 m × 0.2 mm internal diameter (i.d.) fused-silica capillary, coated with cross-linked 5% phenyl methyl silicone (film thickness 0.33 μm). The gas flow rate (helium) was 0.8 ml/min. (split ratio 1:12). The detector and injector temperature were 300 °C, respectively. The oven temperature program was a gradient system: the initial temperature was 120 °C; increased to 270 °C at a rate of 5 °C/min. and held there for 5 min. Then, it was increased to 300 °C at a rate of 10 °C/min and held for 2 min.

2.4. Androgens and corticoids analysis

Urine samples (2 ml), spiked with an internal standard (cholesteryl *iso*-butyrate, 0.2 μg), were applied to a preconditioned Serdolit XAD-2 resin column [19]. After the column was washed with 3 ml of water, androgens were eluted three times with 1 ml of methanol. The eluent was evaporated, then 1 ml of 0.2 M acetate buffer (pH 5.0) and 50 μl of β-glucuronidase/arylsulfatase (from *Helix pomatia*) were added, and the mixture was incubated overnight at 37 °C, or for 3 h at 55 °C. After the solution was cooled to room temperature, 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 dissolved in 50 μl of a silylating-reagent mixture MSHFB/TMCS/TMSIm (2:2:1, by vol.) and heated at 60 °C for 10 min. After heating, the solution was analyzed by GC-MS.

The Hewlett-Packard 5890A gas chromatograph, coupled with a 5970B mass-selective detector with a cross-linked methyl-silicone capillary column (17 m × 0.2 mm i.d., film thickness 0.33 μm) was used. Helium, at a flow rate of 0.85 ml/min, was used as carrier gas, and samples were injected in split mode (split ratio 1:13). The temperature of the injector and the transfer line were set to 280 and 300 °C, respectively. The oven temperature was initially 180 °C, then it was ramped at 25 °C/min to 300 °C, and held for 5 min. The quantitation of the samples was carried out in the selected ion-monitoring (SIM) mode. The dwell time for each ion was set at 50 ms, and the mass spectrometer was operated at 70 eV in the electron-impact (EI) mode.

2.5. Estrogens analysis

The 3 ml urine sample and the 0.5 μg of d_2-estradiol (internal standard) were applied to the column of preconditioned Serdolit XAD-2 resin. After washing with water, the free and conjugated estrogens were eluted with methanol, and the eluant was evaporated to dryness. We performed enzyme hydrolysis using 50 μl of β-glucuronidase/arylsulfatase (from *Helix pomatia*) with 1 ml of 0.2 M acetate buffer (pH 5.0) and ascorbic acid (1 mg/ml) at 37 °C (overnight) or 55 °C for 3 h. After the hydrolysis, potassium carbonate (100 mg) was added, and the mixture was extracted with ethyl acetate. Then, the organic layer was evaporated until dry. The residue was derivatized by the reagent mixture (MSTFA/TMCS, 100:1 volume ratio) at 60 °C for 30 min. After heating, 2 μl aliquots were injected into the GC column by an autosampler.

The Hewlett-Packard GC/MS system consisted of a Model 5890A gas chromatography, a Model 5970B mass-selective detector, and a HP 59970C MS Chemstation. The GC column was a 17 m × 0.2 mm i.d. fused-silica capillary, coated with cross-linked 5% phenyl methyl silicone (film thickness 0.33 μm). The flow rate of carrier gas (helium) was 0.85 ml/min, and the split ratio was 1:13. The GC temperature program was as follows: the initial temperature of 180 °C was increased to 260 °C at a rate of 20 °C/min, and held there for 6 min. Then, it was increased to 275 °C at a rate of 2 °C/min, and held for 8 min. Finally, it was increased to 300 °C at a rate of 15 °C/min. and held for 10 min. The injector temperature was 300 °C, the transfer line was 300 °C, and the ion source was 200 °C. We operated the mass spectrometer at 70 eV in the EI mode. The SIM mode was used for quantifying the estrogen metabolites, and the dwell time for each ion was set at 50 ms.

2.6. Assay

The urinary concentrations of 11 polyamines, 11 estrogens, and 25 androgens and corticoids were measured. All values were corrected for the concentration of creatinine in the same urine. All urine samples were analyzed in separate batches for the 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 polyamine, estrogens, and androgens and corticoids were 48.6–101.2%, 80.97–97.81%, and 72.33–94.54 %, respectively. These results were found to be reproducible and quantitative. The CVs of intraday and interday analysis were 0.03–9.67% and 0.05–10.00% for polyamines, 0.24–10.52% and 1.05–10.24% for estrogens, and 1.43–10.86% and 0.96–9.98% for androgens and corticoids. These methods possess good quality control data [8, 20–21]. Comparison between the cases and the controls was made by a Student's *t*-test and ANOVA

using Statistical analysis Software (SAS, Cary, NC, USA). All directly measured hormone variables were normally distributed, and the statistical significance of the difference in these variables among the three groups (benign and cancer patients and normal controls) were evaluated by ANOVA. With regard to the ratios of benign and cancer cases, statistical analysis for the significance of the difference from the normal value was conducted by the *t*-test. The differences were considered significant at $P < 0.05$.

3. Results

3.1. Urinary profile of polyamines, androgens and corticoids, and estrogens

The levels of 11 urinary polyamines were determined for patients with benign cervical disease and cervical cancer and normal female subjects using gas chromatography nitrogen-phosphorus detection. Table 1 summarizes the concentration levels (mean and range) for the urinary excretion of 11 polyamines.

Most of the polyamine levels of the benign patients were similar to those of the normal subjects; however, for the cancer groups, all 11 polyamine levels were significantly increased ($P < 0.05$) as compared with the normal values. In particular, a noticeable increase of Put, Spd, and Sp levels was observed.

The concentration of 11 estrogen and 25 androgens and corticoids in the urine of patients with benign cervical disease and cervical cancer and normal controls were determined by GC/MS/SIM, and these results are described in Tables 2 and 3, respectively.

Regarding estrogens (Table 2), the levels of major estrogen metabolites (E1, 17α-E2, 17β-E2) and 16-hydroxylation metabolites (16α-OH E1) in the cancer groups were higher than that of normal controls, and benign groups were ranked between them. In particular, the difference between the metabolite level of the cancer patients and the controls was statistically significant at $P < 0.05$. As demonstrated, the controls have a significantly elevated excretion of catechol estrogens (2-MeO E1, 2-OH E1, 2-OH,3-MeO E2, 2-MeO E2, and 4-MeO E2), followed by patients with benign cervical disease and cervical cancer.

As shown in Table 3, the urinary levels of the adrenal-androgen and corticoid metabolites were not significantly different among the three groups. However, the levels of DHEA, Δ^4-Dione, Δ^5-Diol, Te, 11-Keto Et, 11β-OH Et, and THF were increased in the urine of patients with benign and cancer as compared to that of the controls. The excretion of these metabolites in the cervical cancer patients was significantly higher than in those measured in the normal female subjects ($P < 0.05$).

Table 1
Free and acetylated polyamine levels in urine of normal female subjects and patients with benign cervical disease and cervical cancer (μmol/g of creatinine)

Polyamines	Normal ($n = 25$)		Benign ($n = 18$)		Cancer ($n = 18$)		*P*-values	
	Mean	Range	Mean	Range	Mean	Range	Benign	Cancer
1,3-Diacetylpropane	2.93	0.43–5.26	3.30	0.57–6.86	5.21	1.88–10.11	NS[a]	<0.05
Putreacine	1.23	0.01–2.68	3.41	0.52–7.49	23.30	3.28–66.00	NS	<0.01
Cadaverine	1.68	0.06–2.56	1.96	0.19–3.65	3.18	0.40–6.38	NS	<0.05
N-Acetylputrescine	9.73	2.09–21.13	10.42	3.29–31.41	19.45	3.61–53.42	NS	<0.05
N-Acetylcadaverine	3.81	1.02–7.39	3.77	0.51–7.48	5.00	1.15–10.57	NS	<0.05
Spermidine	0.65	0.13–1.95	2.16	0.21–8.34	6.36	1.78–18.90	NS	<0.01
N^8-Acetylspermidine	2.15	0.59–5.41	2.83	0.38–6.14	5.90	0.95–17.64	NS	<0.05
N^1-Acetylspermidine	1.79	0.12–3.62	2.49	0.17–7.55	4.53	1.53–11.36	NS	<0.05
Spermine	0.76	0.07–1.86	1.48	0.09–3.37	4.89	0.17–10.47	NS	<0.01
N^1-Acetylspermine	0.12	0.05–0.56	0.23	0.08–1.46	0.41	0.12–2.45	NS	<0.05
N^1,N^{12}-Diacetylspermine	0.06	0.00–0.21	0.08	0.02–0.27	0.13	0.03–1.24	NS	<0.05

[a] NS, not significant.

Table 2
Estrogen levels of normal female subjects and patients with benign cervical disease and cervical cancer (nmol/g of creatinine)

Estrogens	Normal ($n = 25$)		Benign ($n = 18$)		Cancer t ($n = 18$)		*P*-values	
	Mean	Range	Mean	Range	Mean	Range	Benign	Cancer
17α-Estradiol	nd[a]	nd	1.16	0.55–2.94	3.73	1.41–9.16	–	–
Estrone [E1]	19.67	14.02–24.40	32.85	10.69–63.82	57.61	11.56–119.7	NS[b]	<0.05
17β-Estradiol [E2]	17.57	15.92–21.94	38.76	9.50–79.23	67.74	11.22–182.9	NS	<0.05
2-Methoxy E1 [2-Meo E1]	57.91	13.91–67.91	8.74	2.04–25.56	6.95	2.73–12.52	NS	<0.01
4-Meo E2	22.26	14.58–26.91	1.15	0.47–2.45	1.28	0.31–2.80	NS	<0.01
2-OH E1	112.50	44.62–254.8	55.96	11.21–111.2	27.04	6.52–72.70	NS	<0.05
2-OH, 3-Meo E2	29.96	12.20–37.58	1.29	0.77–2.47	0.92	2.34–1.87	NS	<0.05
16α-OH E1	12.68	9.04–18.32	37.10	5.35–72.66	60.16	9.58–112.1	NS	<0.05
2-Meo E2	19.37	13.83–21.66	4.51	1.65–9.72	1.89	0.90–3.95	NS	<0.01
Estriol [E3]	18.39	8.10–39.24	16.77	2.97–47.00	20.86	2.64–41.72	NS	NS
16-Epi E3	7.36	2.61–18.03	4.59	1.00–14.04	6.95	2.64–15.52	NS	NS

[a] nd, not detected.
[b] NS, not significant.

Table 3
Androgen and corticoid levels of normal female subjects and patients with benign cervical disease and cervical cancer (μmol/g of creatinine)

Androgens and corticoids	Normal ($n = 25$)		Benign ($n = 18$)		Cancer t ($n = 18$)		*P*-values	
	Mean	Range	Mean	Range	Mean	Range	Benign	Cancer
Androsterone [An]	9.26	5.13–17.45	9.35	3.90–14.42	12.41	5.53–26.37	NS[a]	NS
Etiocholanolone [Et]	10.39	4.11–16.45	8.31	3.32–15.14	13.90	4.40–29.96	NS	NS
Dehydroepiandrosterone [DHEA]	1.77	0.34–4.32	3.43	1.31–6.53	7.09	1.18–11.86	NS	<0.05
4-Androstenedione [Δ^4-Dione]	0.91	0.12–2.02	1.12	0.42–3.43	2.12	0.95–4.82	NS	<0.05
5-Androstenediol [Δ^5-Diol]	1.01	0.40–3.17	1.20	0.65–3.20	2.08	0.77–3.51	NS	<0.05
Testosterone [Te]	0.43	0.08–1.43	0.46	0.10–1.43	0.66	0.19–1.82	NS	<0.05
Dihydrotestosterone [DHT]	1.03	0.18–2.87	1.12	0.50–2.35	1.98	0.62–2.90	NS	NS
16α-OH DHEA	3.68	1.00–6.56	3.66	1.15–6.59	3.46	1.07–7.08	NS	NS
5-Androstene-3α,16β,17β-triol [Δ^5-AT]	2.67	1.38–3.14	2.34	0.88–3.92	2.23	1.01–4.30	NS	NS
11-Keto An	1.08	0.14–3.14	1.13	0.06–2.23	1.26	0.07–2.52	NS	NS
11-Keto Et	1.03	0.42–3.58	0.99	0.12–2.00	4.71	1.13–7.28	NS	<0.05
11β-OH An	3.97	1.29–8.52	4.08	2.33–8.24	4.16	2.31–9.02	NS	NS
11β-OH Et	1.78	0.24–2.58	1.84	0.17–2.99	3.24	0.14–5.33	NS	<0.05
Tetrahydrodeoxycorticosterone [THDOC]	0.09	0.03–0.31	0.08	0.02–0.16	0.10	0.03–0.22	NS[a]	NS
Tetrahydro-11-deoxycortisol [THS]	0.49	0.15–1.48	0.27	0.09–0.48	0.37	0.03–0.72	NS	NS
Tetrahydro-11-dehydrocorticosteronel [THA]	0.56	0.08–1.59	0.38	0.12–0.74	0.49	0.11–1.43	NS	NS
Tetrahydrocortisone [THE]	13.36	7.97–25.22	13.53	5.26–20.05	17.88	6.39–34.67	NS	NS
Tetrahydrocortisol [THF]	7.32	3.84–15.55	8.19	4.15–12.52	24.24	7.88–40.04	NS	<0.05
5α-Tetrahydrocortisol [5α-THF]	5.02	1.48–12.76	4.61	2.24–9.19	4.36	0.24–9.84	NS	NS
β-Cortolone	6.00	1.70–9.26	5.88	1.44–8.17	6.70	1.23–9.97	NS	NS
Tetrahydrocorticosterone [THB]	1.17	0.62–3.80	1.58	0.16–3.44	1.56	0.18–3.79	NS	NS
β-Cortolone	1.57	0.99–2.60	1.49	0.17–3.02	2.00	0.40–4.76	NS	NS
β-Cortol	1.48	0.67–2.56	1.53	0.23–2.82	1.55	0.20–3.05	NS	NS
α-Cortol	1.83	0.87–3.36	1.34	0.07–2.44	2.15	0.37–3.72	NS	NS
5α-Tetrahydrocortico sterone [5α-THB]	1.17	0.37–2.00	1.42	0.17–2.87	1.62	0.24–2.25	NS	NS

[a] NS, not significant.

3.2. Relative ratios of polyamines, androgens and corticoids, and estrogens

We compared the concentration ratios of precursor metabolites to product metabolites between normal controls and cancer patients (Table 4) for the following reasons: to discuss a possible involvement of related enzymes in the biomedical pathway of polyamines, estrogens, and androgens and corticoids. We found that the ratios of Put/N^1-acSpd, Spd/N^1-acSd and N^1-acSd/N^1,N^{12}-diacSp for polyamines, 16α-OH E1/2-OH E1 for estrogens and Et/An, 11β-OH Et/11β-OH An, THF/5α-THF and THB/5α-THB for androgens and corticoids were significantly increased in the cervical cancer patients. The increase of the ratios was due to the significant increased level of numerators, polyamine-oxidase metabolites (Put, Spd and N^1-acSp), and 16α-hydroxylation metabolites (16α-OH E1) and the decrease of 5α-reductase metabolites (An, 11-OH An, 5α-THF, 5α-THB).

3.3. Ratios of Put/N^1-acSpd, 16a-OH E1/2-OH E1, and THF/5a-THF for the diagnosis of cervical cancer

Put/N^1-acSpd, 16α-OH E1/2-OH E1, and THF/5α-THF were selected to examine the potential usefulness for cervical-cancer diagnosis. These ratios were combined in normal female subjects and in patients with benign cervical disease and cervical cancer and shown in Fig. 1. In the normal female, the lowest ranges and mean values were observed and these ratios were found to elevate along with the benign and cancer stages. Although no significant difference in the ratios were observed between the benign and control groups, the patients with benign had lower values than did patients with cervical cancer. In particular, the ratios shown in Fig. 1A were more effective to discriminate patients with benign cervical disease and cervical cancer than those shown in (Fig. 1B) and (Fig. 1C).

4. Discussion

Polyamines are now known to be involved in proliferation, differentiation, and neoplastic-transformation, as well as angiogenesis, which is essential for tumor-cell metastasis [7]. Compared to that of normal controls, cancer patients have elevated levels of polyamines in their physiological fluids, such as the serum, plasma and urine [8]. Many reports exist on the usefulness of polyamine levels in the diagnosis of malignant diseases [8,9]. It is also known that

Table 4
Relative ratios of polyamines, estrogens, and androgens and corticoids in urine of normal female subjects and patients with benign cervical disease and cervical cancer

Relative ratios	Normal ($n = 25$)		Benign ($n = 18$)		Cancer t ($n = 18$)		*P*-values	
	Mean	Range	Mean	Range	Mean	Range	Benign	Cancer
Polyamine oxidase (PAO)								
Put/N^1-acSpd	0.49	0.13–1.04	1.37	0.43–3.85	5.14	1.56–9.72	NS[a]	<0.01
Spd/N^1-acSp	5.42	2.11–9.36	9.39	2.88–16.57	15.51	4.69–41.37	NS	<0.05
N^1-acSpd/N^1,N^{12}-diacSp	29.81	4.07–52.91	31.12	5.14–60.21	34.83	5.60–63.45	NS	<0.05
16α-Hydroxylation								
16α-OH E1/2-OH E1	0.15	0.05–0.29	0.66	0.27–1.10	2.23	1.46–3.13	NS	<0.01
5α-Reductase								
Et/An	0.72	0.18–1.76	0.89	0.23–1.85	1.13	1.06–2.72	NS	<0.05
11β-OH Et/11β-OH An	0.23	0.01–0.95	0.45	0.18–1.57	0.78	0.39–2.37	NS	<0.05
THF/5α-THF	0.95	0.09–1.75	1.78	1.67–3.98	5.56	4.84–12.33	NS	<0.01
THB/5α-THB	1.04	0.89–1.19	2.33	2.01–5.14	4.35	3.74–10.32	NS	<0.05

[a] NS, not significant.

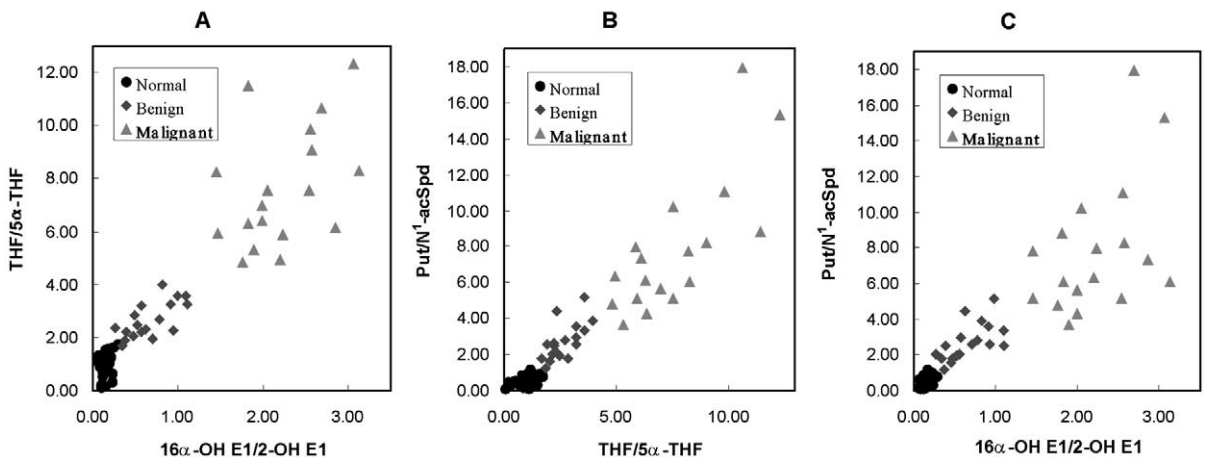

Fig. 1. Typical distributions of the concentration ratio of urinary polyamine, estrogen, and androgen and corticoid in normal female and in patients with benign cervical disease and cervical cancer. (A) 16α-OH E1/2-OH E1 vs. THF/5α-THF, THF/5α-THF vs. Put/N^1-acSpd 16α-OH E1/2-OH E1 vs. Put/N^1-acSpd.

polyamine concentrations rise in the following circumstances: (1) in the serum and urine of patients with non-malignant disease; (2) early stage of cancer; or (3) pregnancy [2,22]. Because of this high false-positive rate, polyamine determinations will play a minor role in cancer screening. In benign diseases or early stages of cancer, only one or two polyamine parameters may show abnormalities. In advanced cancer, however, several polyamine parameters tend to become positive [23]. Therefore, to evaluate a possible role in the screening of cervical cancer, we have determined the concentration of related metabolites such as endogenous steroids, as well as polyamine, in the urine of patients with benign cervical disease and cervical cancer and in normal subjects.

Polyamines and their biosynthetic enzymes are present in larger amounts in the biological fluid of various cancer patients. They are regulated by a complex equilibrium, and numerous enzymes take part in their metabolism [6–9]. Polyamine metabolism in mammalian cells starts at arginine through the action of arginase. The resultant ornithine is converted by one of the key enzymes in polyamine metabolism, ornithine decarboxylase (ODC), to putrescine. Putrescine is then converted to spermidine and spermine [7]. Thus, overexpressed ODC, which provides the precursor for polyamine biosynthesis, confers significant alteration in the polyamine metabolism in the cervical cancer experimental system [6,7]. In cases of human cervical cancers, significantly elevated polyamine levels including Put, Spd, and Sp (Table 1) were found. These results are in good agreement with the previous investigations. Nishioka et al. [7] reported that Spd/Sp ratio of abnormal tissues was lower than that of normal tissues in cervical cancer. In the case of urinary polyamines, our study presented the following: the ratio of urinary Spd to Sp in normal controls was lower than in that of patients with benign cervical disease and cervical cancer. However, the ratio showed no significant difference at $P < 0.05$.

N^1-Acetylspermidine is primarily found in human tumor tissues. Persson et al. [24] observed the increase of N^1-acetylated derivatives of Spd and Sp in mammary-cancer tissues. In this study, the increased urinary concentration of *N*-acetylpolyamines (N^1-acSpd, N^1-acSp and N^1,N^{12}-diacSp) were observed in patients with cervical cancer. However, these differences did not reach the level of statistical significance at $P < 0.05$. N^1-acSpd and N^1-acSp can also be converted to putrescine and spermidine, respectively, by the action of polyamine oxidase [6]. To verify the alteration of PAO activity, we compared the ratios of precursor to product polyamines related to PAOs between normal controls and patients with cervical cancer (Table 4). In the cancer patients, the ratios of Put/N^1-acSpd, Spd/N^1-acSp and N^1-acSpd/N^1,N^{12}-diacSp increased. As a result of these

findings, the following is suggested: the high activity of polyamine oxidase (PAO) leads to elevated urinary concentrations of Put, Spd, and N^1-acSpd, which may be related to the cervical cancer.

Previous studies reported that both HPV and estrogen may play a significant role in the development of cervical cancer [10]. Estrogen alters papillomavirus expression, which is exacerbated during pregnancy when estrogen levels are elevated [12]. Results from animal studies showed that the activity of enzymes in the uterus is modulated by the female sex hormones [25].

We performed a profile analysis for 12 estrogen metabolites in the urine of normal female subjects and of benign cervical disease and cervical cancer patients. These finding are shown in Table 2. We also chose to evaluate the extent of estradiol hydroxylation at the C-2 and C-16α position, which are the two primary and competing sites of estrogen-oxidation. Thus, we compared the ratio of 16α-OH E1 to 2-OH E1 with the cases and the corresponding control group (Table 4). The 2-OH E1 is further metabolized to produce the other catechol estrogens such as 2-Meo E1, 4-Meo E2, 2,-OH-3-Meo E2, and 2-Meo E2 [10]. In the cancer patients, those levels including 2-OH E1 are markedly decreased as compared with normal values. On the other hand, major estrogens (E1, E2) and their C-16α metabolite, 16α-OH E1 was significantly increased in comparison to the normal subjects ($P < 0.05$).

Therefore, the ratio of 16α-OH E1/2-OH E1, which is an index of the relative strengths of the two competing oxidative pathways, was significantly higher in patients with cervical cancer than in those found in patients with benign and controls.

Increased conversion of estradiol to 16α-hydroxyestrone, known to be a risk factor for cancer in some other estrogen-sensitive cells [9]. 16α-Hydroxylation and HPV augment each other's effects and promote cell proliferation. Estrogen metabolites by 16α-hydroxylation increase HPV expression, and the transfection and immobilization of cells with HPV increases the 16α-hydroxylation of estradiol [10]. Contrary to 16α-hydroxylation, the alternate pathway of the metabolism of estradiol, 2-hydroxylation has been shown to be associated with a decreased risk of cancer [9–11]. Sepkovic et al. [11] reported that C-2/C-16α hydroxylated estrogen metabolite ratios are useful biochemical markers for cervical-cancer risk and they correlated these ratios with cervical intraepithelial neoplasia.

The following is suggested from these results concerning the individual estrogen metabolites: (1) a decreased level of catechol estrogens share a negative association with cervical cancer; and (2) major estrogens and 16α-OH E1 are inversely associated with the risk of cervical cancer.

Steroid hormones support the oncogenic activities of the papillomavirus genes by several indirect mechanisms [15]. Previous studies showed that steroid hormones interfere with persistent papillomavirus infections on various levels [15]. Swain et al. [26] reported that androgen secretion modifies estrogenic and progestational stimuli to the estrogen sensitive cells such as breast cancer. In this study, the urinary DHEA levels of cancer patients showed the most significant increase. Among the androgen and corticoid metabolites (Table 3), as well as the concentration of DHEA, that of Δ^4-Dione, Δ^5-Diol, Te, 11-keto Et, 11β-OH Et, and THF showed a statistically significant elevation in the patients with cancer as compared with the levels of those individuals in the normal controls ($P < 0.05$). No significant differences in the urinary concentrations of corticosteroids except THF were found. DHEA and Δ^4-Dione are the available precursors of aromatization to estrogen [27]. In this study, DHEA is the most elevated steroid in patients with cervical cancer. From this results, it is suggested that the excessive production of DHEA and Δ^4-dione may have increased the precursor of estrogen-aromatization and elevated estrogen concentrations in cervical carcinogenesis. It was reported that androgens, such as 5α-DHT, may participate the endocrine-regulation of bovine reproductive tissues [17]. Therefore, we also attempted to study the possible effect on various enzyme activities related to the metabolism of endogenous steroids. Regarding 5α-reductase, the ratios of Et/An, 11β-OH Et/11β-OH An, THB/5α-THB, and THF/5α-THF were compared, and it was found that they were significantly increased in the patients with cervical cancer ($P < 0.05$).

Finally, we wanted to determine the relationship between the activity of enzymes and estimate the possible usefulness of a disease screening marker. Thus, we combined the individual ratios of PAO,

16α-hydroxylation, and 5α-reductase metabolites. The urinary Put/N^1-acSpd, 16α-OH E1/2-OH E1 and THF/5α-THF ratios were selected because these ratios were the most highly changed polyamines, estrogens, and androgens and corticoid metabolites in cervical-cancer patients ($P < 0.01$). Fig. 1 demonstrated that the patients with cervical cancer could be differentiated from the patients with benign cervical disease as well as from the normal controls. The results of the linear correlation analysis of 16α-OH E1/2-OH E1 vs. THF/5α-THF ($r = 0.847, P < 0.01$), THF/5α-THF vs. Put/N^1-acSpd ($r = 0.855, P < 0.05$) and 16α-OH E1/2-OH E1 vs. Put/N^1-acSpd ($r = 0.752, P < 0.05$) represented a positive relationship in patients with cervical cancer. In particular, the ratios shown in Fig. 1A distinctly demonstrate that the difference between benign and cervical cancer patients was statistically significant ($P < 0.01$). However, the ratios shown in Fig. 1B,C were more effective in discriminating cervical cancer rather than benign disease. Thus, it is suggested that the combination of 16α-OH E1/2-OH E1 and THF/5α-THF may be worthy for predicting the possible risk of cervical cancer.

In conclusion, our study suggests the following: (1) the increase of 16α-hydroxylation in estrogen metabolism; (2) the high activity of polyamine oxidase (PAO) in polyamine metabolism; and (3) the low activity of 5α-reductase in androgen metabolism may play a significant role in the development of cervical cancer. Although further research is necessary, the combination of 16α-OH E1/2-OH E1 and THF/5α-THF may be a useful dual marker for the discrimination of patients with benign cervical disease and cervical cancer and normal controls.

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