both donor and recipient mice were subjected to ovariectomy. These mice were injected s.c. with 100 μg/kg estradiol valerate (Nihon Schering, Osaka, Japan) in corn oil every week from the time of ovariectomy. Two weeks after ovariectomy, donor mice were killed. Uterine horns were removed and put into a dish containing phosphate-buffered saline (PBS). Endometrium-rich fragments, obtained by peeling off the serosa and myometrium, were finely chopped using a razor blade. Fragments suspended in 0.6 ml PBS were injected with an 18-gauge needle through the abdominal wall just below the umbilicus into the peritoneal cavity of recipient mice with the ratio of a donor to two recipients. The experiments were performed twice independently, preparing 13 and 14 recipient mice for further treatment. In sham-operated mice, 0.6 ml PBS without fragments was injected. All procedures were conducted under aseptic conditions.

2.3. FR 167653 treatment

The recipient mice underwent subcutaneous injection of vehicles or FR 167653 (kindly provided by Fujisawa Pharmaceutical, Osaka, Japan) at a dose of 30mg/kg dissolved in 200 μl physiological saline twice every day, starting 2 days before the injection of the endometrial fragments and lasting for 23 days. The dose of FR 167653 was chosen with reference to papers showing in vivo effects of the drug (Hatanaka et al., 2001; Nishikori et al., 2002; Takahashi et al., 2001).

The mice were killed through cervical dislocation on the day after the last injection day.

2.4. Collection of the peritoneal fluid and the endometriotic tissues

After sacrifice, PBS (0.8 ml) was injected into the peritoneal cavity of each mouse. After vigorous shaking of the mice, peritoneal fluid was collected. Peritoneal fluids were centrifuged and the supernatant was kept at −80 °C until assay. Sediment peritoneal cavity cells, which include peritoneal macrophages, were used for RNA extraction. Then, laparotomy was performed, and the number of endometriotic foci was counted. Each focus was excised to exclude as much normal surrounding tissues as possible, and the weight of the excised tissues was measured. In the case of cystic lesions, fluid contents were excluded before the measurement. The tissue of each focus was histologically confirmed as endometriotic lesions. In one experiment, the uterus was excised to measure its weight. In all the procedures, examiners were blinded to the treatment given to each mouse.

2.5. RNA extraction and quantitative real-time RT-PCR

Real-time quantitative PCR was performed as we had previously described (Hirota et al., 2003). RNA was extracted from endometriotic lesions and peritoneal cavity cells using ISOGEN (Wako, Osaka, Japan). Total RNA (0.7 μg) was reverse-transcribed in a 20 μl volume using Rever Tra Ace (TOYOBO, Tokyo, Japan). To assess IL-6 and MCP-1 mRNA expression, real-time quantitative PCR and data analyses were performed using a Light Cycler (Roche Diagnostic GmbH, Mannheim, Germany), according to the manufacturer's instructions. Expression of IL-6 and MCP-1 mRNA was normalized to

RNA loading for each sample using GAPDH mRNA as an internal standard. The following PCR primers were used; IL-6 primers (sense, 5′-TGTGCAATGGCAATTCTGAT-3′; antisense, 5′-GGAAATTGGGGTAGGAAGGA-3′) MCP-1 primers (sense, 5′-AGCCAGCTCTCTCTTCCTCC-3′; antisense, 5′-TCTGGACCCATTCCTTCTTG-3′) GAPDH primer (sense, 5′-ACCACAGTCCATGCCATCAC-3′; antisense, 5′-TCCACCACCCTGTTGCTGTA-3′). PCR conditions of IL-6 and MCP-1 for amplifications were 40 cycles at 95 °C for 15 s, 64 °C for 10 s, and 72 °C for 12 s, followed by melting curve analysis. PCR conditions of GAPDH for amplifications were 40 cycles at 95 °C for 15 s, 64 °C for 10 s, and 72 °C for 20 s, followed by melting curve analysis. Each PCR product was purified with a QIAEX II gel extraction kit (QIAGEN, Tokyo, Japan), and their identities were confirmed using an ABI PRISM^{TM} 310 genetic analyzer (Applied Biosystems, Foster City, CA).

2.6. Measurement of cytokine

Concentrations of IL-6 and MCP-1 in peritoneal fluids were measured using respective mouse specific enzyme-linked immunosorbent assays (ELISA) (Genzyme/Techne, Minneapolis, MN, USA). The minimum detectable doses were 10 and 7.8 pg/ml for IL-6 and MCP-1, respectively. The intra-assay and inter-assay coefficients of variation were less than 5% in these assays.

2.7. Statistical analysis

Data are expressed as mean ± S.E.M. The unpaired Student's *t*-test was used for comparisons. Statistical significance was defined as $p < 0.05$.

3. Results

Endometriotic lesions developed in the mouse abdomen and collected specimens are shown in Fig. 1. In Fig. 2, total weights of all the endometriotic lesions per mouse are shown. The total weights were significantly low in the FR 167653-treated mice (5.2 ± 0.6 mg) as compared to the control mice (12.6 ± 1.5 mg, $p < 0.0005$). In sham-operated mice, no endometriotic lesion was observed in the peritoneal cavity.

Concentrations of IL-6 and MCP-1 in peritoneal fluids are shown in Fig. 3. The concentrations were both remarkably increased by the induction of endometriosis, while the increases were both suppressed with FR 167653 treatment ($p < 0.01$ for IL-6, $p < 0.05$ for MCP-1).

The expression levels of IL-6 mRNA were decreased both in endometriotic lesions and in peritoneal cavity cells of FR167653-treated mice as compared to those of the control mice (Table 1). As for MCP-1 mRNA, the expression levels were significantly decreased in peritoneal cavity cells, but not in endometriotic lesions.

The number of endometriotic lesions per mouse, weight of the uterus and the body are shown in Table 2. Respective values were essentially the same between the FR 167653-treated group and the control group.

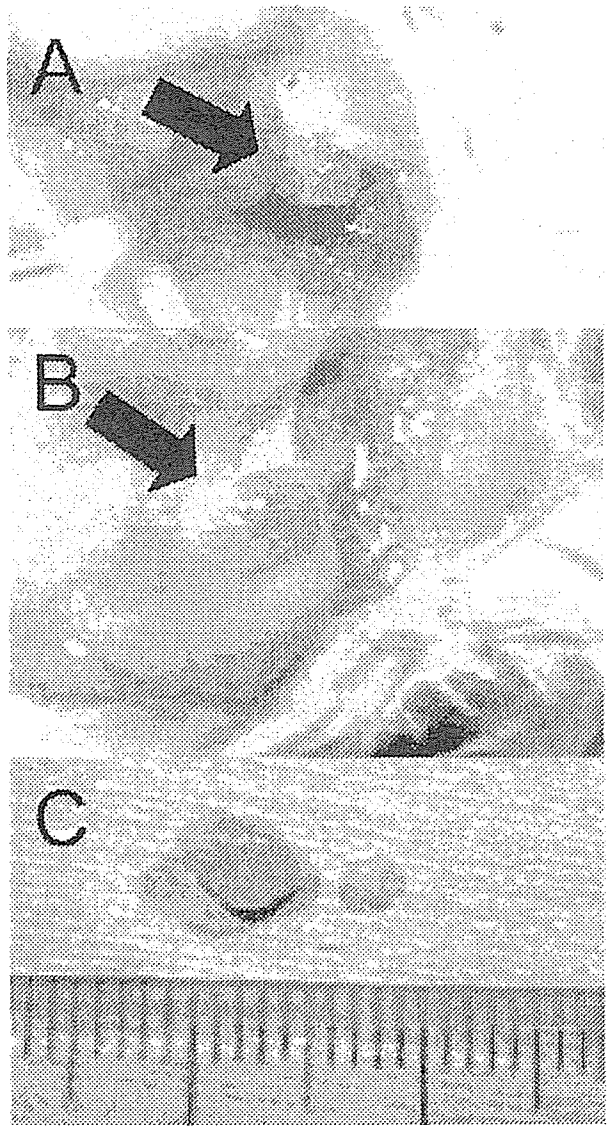

Fig. 1. (A and B) Endometriotic lesions developed in the mouse abdomen (arrows); (C) excised specimens of endometriotic lesions in (A) and (B).

Table 1
Interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 mRNA expression in endometriotic lesions and peritoneal cavity cells

	Endometriotic lesions		Peritoneal cavity cells	
	C	FR	C	FR
IL-6	1.00 ± 0.19	0.44 ± 0.17*	3.07 ± 0.68	1.38 ± 0.20*
MCP-1	1.00 ± 0.12	0.87 ± 0.15	0.23 ± 0.04	0.11 ± 0.01*

The values shown are mean ± S.E.M., as relative ratio to endometriotic lesions in the control mice. The data of two independent experiments were combined. C, control mice; FR, FR167653-treated mice.
* $p<0.05$ vs. control.

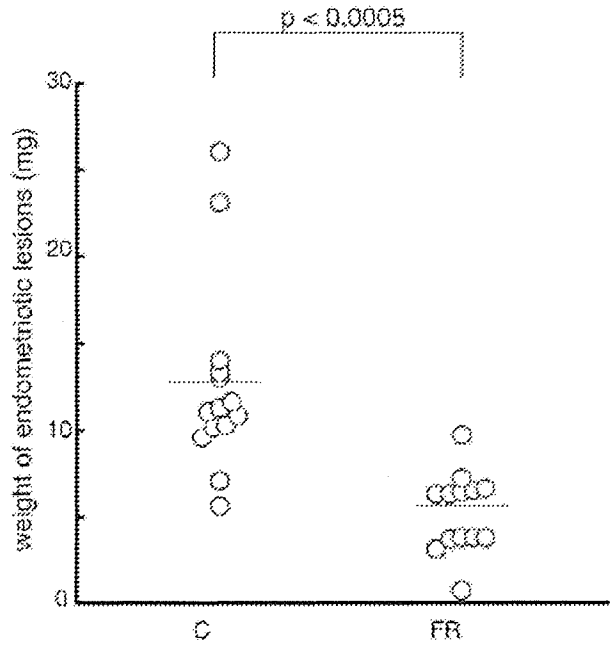

Fig. 2. Total weight of endometriotic lesions in murine model of endometriosis. Endometriosis was induced in BALB/c mice by injecting syngenic endometrial fragments into the peritoneal cavity. Thirteen mice were treated with FR 167653 (FR) 30 mg/kg, s.c. twice per day starting 2 days before the endometrial injection and lasting for 23 days, while 14 mice used as controls (C) were treated with vehicles. The data of two independent experiments were combined. Horizontal bars represent the means of each group.

Table 2
The number of endometriotic lesions, the body weight and the uterine weight

	C	FR	
Number of lesions[a]	2.8 ± 1.2	2.6 ± 1.1	N.S.
Uterine weight[b] (mg)	159 ± 74	130 ± 12	N.S.
Body weight[a] (g)	19.2 ± 1.9	19.1 ± 1.2	N.S.

C, control mice; FR, FR167653-treated mice; N.S., not significant.
[a] The data of two independent experiments were combined.
[b] Weight of the uterus was measured in one experiment.

4. Discussion

In the present study, we demonstrated that the treatment with FR 167653, a p38 MAPK inhibitor, decreased the weight of experimentally induced endometriotic lesions. In parallel with this finding, the concentrations of IL-6 and MCP-1 in peritoneal fluid, which were elevated in mice harboring endometriosis, decreased.

A framework of current concept on endometriosis is that inflammatory status in the pelvic cavity of women with the disease are activated with increasing levels of proinflammatory cytokines, which promote the growth of endometriotic lesions (Lebovic et al., 2001). The inflammatory reactions are suggested to be self-perpetuating by feed-forward interactions among endometriotic cells and peritoneal cavity cells, particularly, peritoneal

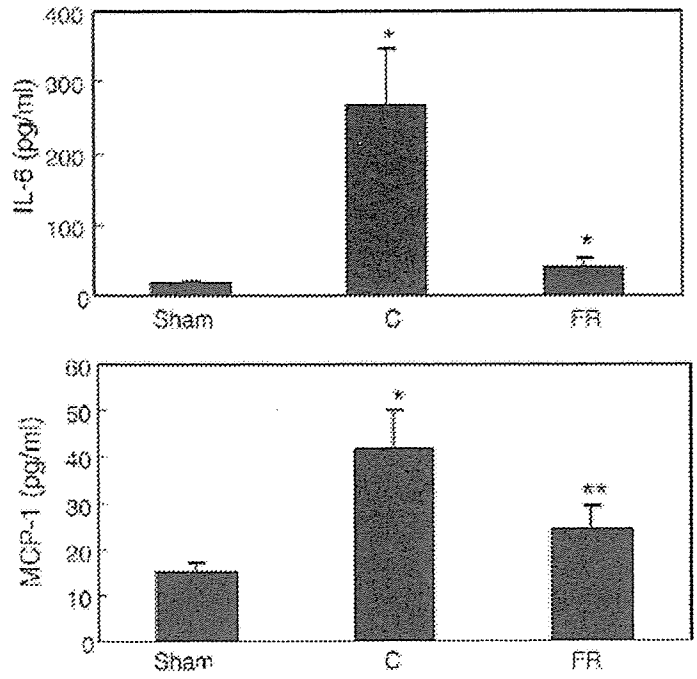

Fig. 3. Concentrations of interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 in peritoneal fluids in murine model of endometriosis. Endometriosis was induced in BALB/c mice by inoculating syngenic endometrial fragments. The sham-operated group (Sham) was inoculated phosphate-buffered saline (PBS). Mice for the induction of endometriosis were treated with vehicles (C) or FR 167653 (FR) 30 mg/kg, s.c. twice per day starting 2 days before the endometrial injection. IL-6 and MCP-1 concentrations in peritoneal fluids were measured using specific ELISA. The combined data of two separate experiments are shown. Values are shown with mean ± S.E.M. *, $p<0.01$ vs. C; **, $p<0.05$ vs. FR.

macrophages. In this respect, it is a notable finding that FR 167653 treatment decreased the mRNA expression of proinflammatory cytokine(s) both in endometriotic lesions and in peritoneal cavity cells. This downregulation might lead to subsidence of the intraperitoneal inflammation raised by the development of endometriosis. Indeed, the concentrations of IL-6 and MCP-1 in the peritoneal fluid were increased in endometriotic mice as compared to non-endometriotic mice, while the increase in endometriotic mice were suppressed by FR 167653 treatment. Therefore, in light of the current concept, the inhibition of the growth of endometriotic lesions observed in FR 167653-treated mice may in part be related to the suppression of peritoneal inflammation.

Several steps are surmised in the formation of endometriotic lesions, starting from intraperitoneal dispersal of endometrial cells with the retrograde menstrual blood. Among others, two major steps are the peritoneal implantation of the refluxed cells and the growth of established endometriotic lesions, in which different molecules are suggested to be functioning (Kyama et al., 2003). In the present study, the number of endometriotic lesions was comparable even though the commencement of FR 167653 treatment antedated injection of endometrial fragments, whereas the growth of the lesions was significantly suppressed with the treatment. The finding implies that the inhibitory effect of FR 167653 might be exerted mainly in the growth phase rather than in the implantation phase of the progress of endometriosis.

Another remarkable finding is that FR 167653 treatment effected a slowdown in the growth of endometriotic lesions without changing the uterine weight. The precise reason for the differential susceptibility to FR 167653 is unknown. The increased activation levels of p38 MAPK in endometriotic lesions as compared to eutopic endometrium (Yoshino et al., 2004) may partly explain the observed difference.

Endometriosis-associated intraperitoneal inflammation is supposed to impair fecundity (Harada et al., 2001). The decrease in the levels of IL-6 and MCP-1 in peritoneal fluid observed in the FR 167653-treated mice may reflect an improved inflammatory environment in the peritoneal cavity, and imply therapeutic potential of FR 167653 for infertility associated with endometriosis.

The therapeutic effect of anti-inflammatory drugs for endometriosis was suggested by a report documenting that pentoxyfylline reduced endometriotic implant growth in a rat endometriosis model (Nothnick et al., 1994). FR 167653 is a novel anti-inflammatory drug with a distinct spectrum of activities as compared to anti-inflammatory steroids or non-steroidal anti-inflammatory drugs (Hatanaka et al., 2001; Nishikori et al., 2002). In addition, a novelty of application of FR 167653 for endometriosis therapy is that it is targeted to p38 MAPK, a possible causative molecule of endometriosis.

In summary, we demonstrated that FR 167653, a p38 MAPK inhibitor, decreased the weight of endometriotic lesions in a murine model. The effect appeared to be related with the suppression of intraperitoneal inflammation associated with endometriosis.

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References

Dabrosin, C., Gyorffy, S., Margetts, P., Ross, C., Gauldie, J., 2002. Therapeutic effect of angiostatin gene transfer in a murine model of endometriosis. Am. J. Pathol. 161, 909–918.

Harada, T., Iwabe, T., Terakawa, N., 2001. Role of cytokines in endometriosis. Fertil. Steril. 76, 1–10.

Hatanaka, K., Kawamura, M., Murai, N., Ogino, M., Majima, M., Ogino, K., Harada, Y., 2001. FR167653, a cytokine synthesis inhibitor, exhibits anti-inflammatory effects early in rat carrageenin-induced pleurisy but no effect later. J. Pharmacol. Exp. Ther. 299, 519–527.

Hirota, Y., Osuga, Y., Yoshino, O., Koga, K., Yano, T., Hirata, T., Nose, E., Ayabe, T., Namba, A., Tsutsumi, O., Taketani, Y., 2003. Possible roles of thrombin-induced activation of protease-activated receptor 1 in human luteinized granulosa cells. J. Clin. Endocrinol. Metab. 88, 3952–3957.

Kawano, T., Ogushi, F., Tani, K., Endo, T., Ohmoto, Y., Hayashi, Y., Sone, S., 1999. Comparison of suppressive effects of a new anti-inflammatory compound, FR167653, on production of PGE2 and inflammatory cytokines, human monocytes, and alveolar macrophages in response to endotoxin. J. Leukoc. Biol. 65, 80–86.

Kyama, C.M., Debrock, S., Mwenda, J.M., D'Hooghe, T.M., 2003. Potential involvement of the immune system in the development of endometriosis. Reprod. Biol. Endocrinol. 1, 123.

Lebovic, D.I., Mueller, M.D., Taylor, R.N., 2001. Immunobiology of endometriosis. Fertil. Steril. 75, 1–10.

Maruyama, M., Osuga, Y., Momoeda, M., Yano, T., Tsutsumi, O., Taketani, Y., 2000. Pregnancy rates after laparoscopic treatment Differences related to tubal status and presence of endometriosis. J. Reprod. Med. 45, 89–93.

Matsuyama, W., Wang, L., Farrar, W.L., Faure, M., Yoshimura, T., 2004. Activation of discoidin domain receptor 1 isoform b with collagen up-regulates chemokine production in human macrophages: role of p38 mitogen-activated protein kinase and NF-kappa B. J. Immunol. 172, 2332–2340.

Momoeda, M., Taketani, Y., Terakawa, N., Hoshiai, H., Tanaka, K., Tsutsumi, O., Osuga, Y., Maruyama, M., Harada, T., Obata, K., Hayashi, K., 2002. Is endometriosis really associated with pain? Gynecol. Obstet. Invest. 54 (Suppl. 1), 18–21.

Nishikawa, M., Myoui, A., Tomita, T., Takahi, K., Nampei, A., Yoshikawa, H., 2003. Prevention of the onset and progression of collagen-induced arthritis in rats by the potent p38 mitogen-activated protein kinase inhibitor FR167653. Arthritis. Rheum. 48, 2670–2681.

Nishikori, T., Irie, K., Suganuma, T., Ozaki, M., Yoshioka, T., 2002. Anti-inflammatory potency of FR167653, a p38 mitogen-activated protein kinase inhibitor, in mouse models of acute inflammation. Eur. J. Pharmacol. 451, 327–333.

Nothnick, W.B., Curry, T.E., Vernon, M.W., 1994. Immunomodulation of rat endometriotic implant growth and protein production. Am. J. Reprod. Immunol. 31, 151–162.

Ono, K., Han, J., 2000. The p38 signal transduction pathway: activation and function. Cell Signal. 12, 1–13.

Somigliana, E., Vigano, P., Rossi, G., Carinelli, S., Vignali, M., Panina-Bordignon, P., 1999. Endometrial ability to implant in ectopic sites can be prevented by interleukin-12 in a murine model of endometriosis. Hum. Reprod. 14, 2944–2950.

Takahashi, S., Keto, Y., Fujita, T., Uchiyama, T., Yamamoto, A., 2001. FR167653, a p38 mitogen-activated protein kinase inhibitor, prevents *Helicobacter pylori*-induced gastritis in *Mongolian gerbils*. J. Pharmacol. Exp. Ther. 296, 48–56.

Yamamoto, N., Sakai, F., Yamazaki, H., Nakahara, K., Okuhara, M., 1996. Effect of FR167653, a cytokine suppressive agent, on endotoxin-induced disseminated intravascular coagulation. Eur. J. Pharmacol. 314, 137–142.

Yoshino, O., Osuga, Y., Hirota, Y., Koga, K., Hirata, T., Harada, M., Morimoto, C., Yano, T., Nishii, O., Tsutsumi, O., Taketani, Y., 2004. Possible pathophysiological roles of mitogen-activated protein kinases (MAPKs) in endometriosis. Am. J. Reprod. Immunol. 52, 306–311.

Yoshino, O., Osuga, Y., Hirota, Y., Koga, K., Hirata, T., Yano, T., Ayabe, T., Tsutsumi, O., Taketani, Y., 2003. Endometrial stromal cells undergoing decidualization down-regulate their properties to produce proinflammatory cytokines in response to interleukin-1beta via reduced p38 mitogen-activated protein kinase phosphorylation. J. Clin. Endocrinol. Metab. 88, 2236–2241.

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Elevated Serum Bisphenol A Levels under Hyperandrogenic Conditions may be Caused by Decreased UDP-glucuronosyltransferase Activity

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Abstract. This study was performed to investigate the effect of androgen on the metabolism of bisphenol A (BPA), an endocrine disruptor, in order to clarify the mechanism of the higher levels of serum BPA in men and hyperandrogenemic women compared with normal women. Castrated female rats (OVX) were subcutaneously injected with testosterone propionate (TP) (0.01, 0.1, and 1 mg) every day for 2 weeks. Serum BPA concentrations in OVX rats showed a TP dose-dependent increase and were significantly higher at 0.1 and 1.0 mg of TP. The enzyme reaction of BPA glucuronidation in the rat liver microsomes showed that the ratio of glucuronide in the OVX rats was significantly reduced in a TP dose-dependent manner. Analysis of the mRNA expression of UDP-glucuronosyltransferase 2B1 (UGT2B1) by real-time quantitative RT-PCR revealed that the relative expression level of UGT2B1 mRNA showed a TP dose-dependent decrease. The results of enzyme analyses demonstrated that the ratio of BPA glucuronidation and the expression level of UGT2B1 mRNA were significantly lower under the hyperandrogenemic conditions. The clearance of BPA may be slowed in a TP dose-dependent manner, resulting in an increase of serum BPA concentration under hyperandrogenemic conditions.

Key words: Bisphenol A, UDP-glucuronosyltransferase, UGT2B1, Endocrine disruptor, Androgen

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THERE is substantial evidence that endocrine-disrupting chemicals have the potential to alter the normal function of the endocrine system in wildlife and humans [1, 2]. Various environmental chemicals are known to act as endocrine disruptors, which have been reported to exhibit estrogenic, anti-estrogenic and/or anti-androgenic actions in wildlife and human beings. There is increasing scientific concern and public debate about the involvement of endocrine-disrupting chemicals in a number of human health disorders.

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Bisphenol A (BPA), an estrogenic endocrine-disrupting chemical with two unsaturated phenol rings, is widely used in the production of polycarbonate plastics and epoxy resins, which are used in dentistry, food packaging, and as lacquers for coating food cans, bottle tops and water pipes [3, 4]. A significant amount of BPA has been detected in liquid from canned vegetables that are exposed to high temperature during autoclaving [5], and in the saliva of dental patients fitted with restorative materials [6]. BPA has been reported to bind to estrogen receptors (ERα and ERβ) and to play either estrogenic or anti-estrogenic roles *in vitro* [7, 8]. BPA has been shown to exhibit several actions, such as uterotrophic effects [9], decreasing sperm production [10], stimulation of prolactin release [11], promotion of cell proliferation in a breast cancer cell

line [5], and influencing preimplantation development [12], in animal experiments. Promotion of growth and puberty by fetal or preimplantation exposure to BPA in mice has been reported [13, 14]. We reported that BPA was detectable not only in the serum but also in several biological fluids, including amniotic fluid, demonstrating human fetal exposure to BPA through the placenta [15]. It is interesting to note that there was a gender difference in serum BPA concentrations in humans [16] and, more recently, determination of serum BPA concentrations in women with or without ovarian dysfunction revealed that BPA levels may vary according to the endocrinological status of subjects [17]. BPA levels in patients with endometrial hyperplasia and endometrial cancer, both of which are estrogen-related uterine disorders, were lower compared to those in normal controls [18]. These findings suggest an association between BPA exposure and endocrine disorders. The mode of action of BPA may be even more complex than expected, but analysis of BPA metabolism in the body may provide clues about the mechanisms of linkage between the occurrence of estrogen-related diseases and endocrine disruption.

The glucuronidation reaction is catalyzed by members of the UGT enzyme superfamily [19]. The UGT enzymes are classified into two families, UGT1 and UGT2, on the basis of amino acid sequence homology; the latter is further subdivided into the subfamilies UGT2A and UGT2B. Moreover, UGT2B is subdivided into many isoenzymes. Each isoenzyme has specific substrate(s) for glucuronidation. BPA is known to be glucuronidated by liver microsomes in a reaction catalyzed by UGT2B1, and then to be rapidly excreted in the feces and urine [20–22]. We reported that the ratio of BPA glucuronidation and the expression level of UGT2B1 mRNA were significantly higher in female than in male rat livers [23].

In this study, we investigated the effect of androgen on the metabolism of BPA in rats, in order to clarify the mechanism of the sex difference in serum BPA levels and of the higher levels of serum BPA in patients with polycystic ovary syndrome compared with normal women.

Materials and Methods

Reagents

Bisphenol A (>95% pure) was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). Testosterone propionate, uridine 5'-diphosphoglucuronic acid, Tris-HCl buffer, bovine serum albumin (BSA), EDTA, sodium carbonate, sodium citrate, and Folin-phenol reagent were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sucrose was obtained from ICN Biomedicals, Inc. (Aurora, OH, USA). Magnesium chloride, potassium chloride, methanol, ethanol, copper sulfate pentahydrate, β-glucuronidase, and dimethylbutylidene-bisphenol (internal standard for HPLC) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Acetonitrile and acetic acid were purchased from Kanto Kagaku Co. (Tokyo, Japan).

Animals and their experimental preparation

Eight-week-old female Wistar-Imamichi rats, which were obtained from Saitama Experimental Animals Supply Co., Ltd. (Saitama, Japan), were castrated. Castrated female rats (OVX) were subcutaneously injected with testosterone propionate (TP) (0.01 mg, 0.1 mg, 1 mg; n = 5, respectively) in 0.5 ml of corn oil every day for 2 weeks starting on the fourth day after the castration. The experimental groups consisted of intact rats, ovariectomized rats without TP (OVXTP-0), ovariectomized with TP 0.01 mg (OVXTP-0.01), ovariectomized with TP 0.1 mg (OVXTP-0.1) and ovariectomized with TP 1 mg (OVXTP-1). The rats in intact control and OVXTP-0 groups were subcutaneously injected with 0.5 ml of corn oil. The rats were housed in a controlled-climate room at 22 ± 2°C and 62.5 ± 2.5% relative humidity, with lights on from 08.00 to 20.00 h. They were allowed free access to a standard rodent diet and tap water in glass bottle. Wood chips were used as bedding. Blood samples were obtained by decapitation, and all sera were stored at −30°C until assayed. Livers were removed promptly and stored at −80°C until used.

Serum BPA concentrations in each group of animals (n = 5, respectively) were determined before and 120 min after subcutaneous injection with BPA in corn oil (50 mg/kg). In some experiments, serum BPA concentrations were measured before and at 60, 120, 180 min after the treatment with BPA.

Measurement of rat serum BPA, testosterone and estradiol concentrations

Rat serum BPA concentrations were assayed with a

competitive ELISA [24]. This assay method measures total (conjugated and unconjugated) BPA. Serum testosterone and estradiol concentrations were assayed with ^{125}I-RIA kits (Diagnostic Products Corporation, Los Angeles, CA, USA). All assays were performed in duplicate, and all of the samples were assayed simultaneously. The intra-assay coefficient of variation was less than 10%.

Preparation of liver microsomes and enzyme activity analysis

Livers were perfused with 5 ml of ice-cold physiological saline, and homogenized with five volumes of 50 mM Tris/HCL buffer (pH 7.5) containing 0.25 M sucrose, 25 mM KCl, and 5 mM $MgCl_2$, using a glass-teflon homogenizer. The homogenates were centrifuged at 1000 g for 10 min, and the supernatants were centrifuged at 12000 g for 20 min, and the resultant supernatants were centrifuged at 105000 g for 60 min. The cytosolic supernatants were removed, and the remaining microsomal pellets were suspended in 50 mM Tris/HCl buffer containing 0.25 M sucrose, and stored at −80°C until used. The protein content was determined by the method of Lowry *et al.* [25] using BSA as a standard.

One milligram of microsomal protein was incubated in a total volume of 2.0 ml of 0.1 M Tris/HCl buffer (pH 7.2) containing 1 mM BPA, 0.1 mM UDP-glucuronic acid, 10 mM $MgCl_2$, and 20 μM EDTA. Reactions were performed at 37°C with shaking (frequency: 100 cycles/min; amplitude: 3.5 cm) for 30 min. The reaction was terminated by the addition of 100 μl of methanol, and proteins were removed by centrifugation at 1000 g for 15 min. The supernatants were stored as the reaction products at −80°C until assayed.

Measurement of glucuronide fraction of BPA in the microsomal reaction products

The microsomal reaction supernatant sample was divided in two aliquots for measuring total and unconjugated BPA. After 100 μl of the supernatant sample was washed with 1 ml of a mixture of n-hexane and ethylether (3 : 2), the unconjugated fraction was eluted in the solvent. An aliquot (20 μl) of the solvent fraction was evaporated under a stream of nitrogen gas. An aliquot (1.25 ml) of 0.1 M Tris/HCl buffer containing 1 M $MgCl_2$ and 0.5 mM EDTA was added to the dry residue in the test tube. This sample was used for assaying unconjugated BPA. The procedure for the measurement of total BPA was done as follows. The above-mentioned Tris buffer (1 ml) was added to an aliquot (100 μl) of the supernatant sample, and then 1.23 ml of the Tris buffer was added to 20 μl of the mixture. This sample was used for assaying total BPA by ELISA, which measured both conjugated and unconjugated BPA. This method was selected after comparison with an HPLC technique [23]. There was a significant correlation ($r = 0.877$, $p<0.001$) in the results between this extraction method and the HPLC assay.

RT-PCR analysis of UGT2B1 mRNA

Real-time quantitative RT-PCR was used for the quantification of UGT2B1 mRNA. In brief, 1 μg of total RNA was prepared from rat liver using a modified acid guanidium thiocyanate-phenol-chloroform method. The total RNA treated with RNase-free DNase (Boehringer Mannheim, Mannheim, Germany) was subjected to reverse transcription using oligo dT 20 (Takara, Tokyo, Japan) and superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA) at 42°C for 30 min, followed by RNase H treatment. Aliquots of the cDNA (1/20) were used as templates for real-time PCR analysis using a LightCycler system (Roche, Mannheim, Germany). The PCR program consisted of 40 cycles of 8 sec at 94°C, 5 sec at 60°C, and 10 sec at 72°C. Oligonucleotide primers for UGT2B1 were as follows: forward, 5'-TGTTGGTAT TCCCTTGTTTGC-3'; reverse, 5'-GTGCTTGGCTC CTTTGTGACG-3'. The primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), housekeeping gene, were as follows: forward, 5'-ACCACAGTC CATGCCATCAC-3'; reverse, 5'-TCCACCACCCT GTTGCTGTA-3'. The RNA preparation and RT-PCR in the present study were performed in triplicate.

Statistical analyses

All results are expressed as means ± standard errors of the mean (SEM). Statistical comparisons among the groups were performed by analysis of variance (ANOVA) and the least significant difference test (LSD). Correlation coefficients were calculated by linear regression analysis. Significance was taken at $p<0.05$.

Results

Table 1 shows serum BPA, testosterone and estradiol concentrations in the intact and OVX rats. The serum BPA concentrations were significantly higher in the OVXTP (0.1) and OVXTP (1) groups compared with the OVXTP (0) group. The serum testosterone concentrations were decreased by half in the OVXTP (0) control rats, and were significantly higher in a dose-dependent manner in OVX rats treated with TP. The serum estradiol concentrations in the OVX rats were decreased to less than half the control levels. There was a significant positive correlation between the serum BPA and testosterone concentrations ($r = 0.983$, $p<0.001$), but not between the serum BPA and estradiol concentrations ($r = -0.032$, NS) in all rats.

Subcutaneous injection of BPA (50 mg/kg) into intact female rats caused an abrupt increase of serum BPA concentrations, which reached a peak (749.6 ± 113.8 ng/ml) at 120 min after the treatment, as shown in Fig. 1. Castrated female rats were also subcutaneously injected with BPA (50 mg/kg), and blood samples were obtained at 120 min after the treatment with BPA. The serum BPA concentrations increased in a TP dose-dependent manner, and were significantly higher in the OVXTP (0.1) and OVXTP (1) groups compared with the OVXTP (0) group (Fig. 2).

The levels of total, unconjugated, and conjugated BPA, and the ratios of conjugated to total BPA in the supernatant of the rat liver microsome reaction are listed in Table 2. There were no differences in the total levels of BPA in the reaction products among the groups. The ratio of conjugated to total BPA was significantly lower ($P<0.05$) in the OVXTP (0.1) and OVXTP (1) groups than in the OVXTP (0) or intact control group.

The relative expression level of GAPDH mRNA was not significantly different among the groups, but that of UGT2B1 mRNA was significantly lower ($P<0.05$) in

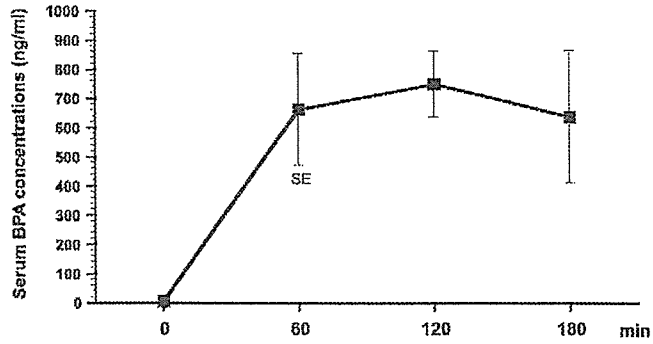

Fig. 1. Serum BPA concentrations in intact female rats after subcutaneous injection of BPA (50 mg/kg).
Subcutaneous injection of BPA into intact female rats caused an abrupt increase of serum BPA concentrations, which reached a peak at 120 min after the treatment ($n = 5$, respectively).

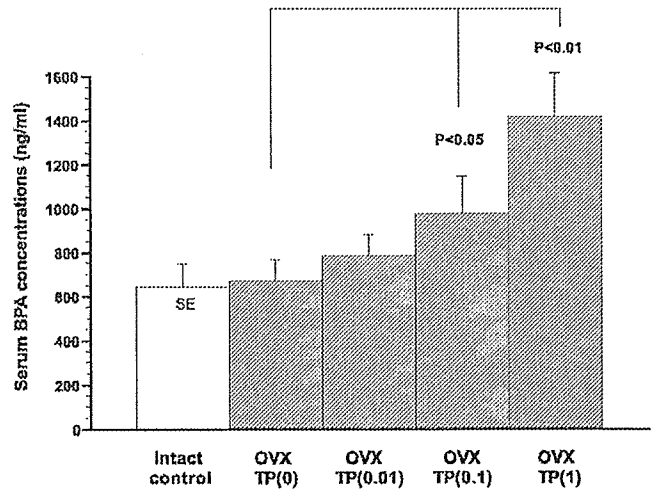

Fig. 2. Serum BPA concentrations in female intact control, ovariectomized (OVX) control, and OVX rats with testosterone propionate (TP) at 120 min after subcutaneous injection of BPA (50 mg/kg).
The serum BPA concentrations increased in a TP dose-dependent manner, and were significantly higher in the OVXTP (0.1) and OVXTP (1) groups compared with the OVXTP (0) group. Values are mean ± SEM ($n = 5$ in each group).

Table 1. Basal serum BPA, testosterone and estradiol concentrations in intact and ovariectomized (OVX) rats

	Intact control	OVXTP (0)	OVXTP (0.01)	OVXTP (0.1)	OVXTP (1)
BPA (ng/ml)	4.38 ± 0.34	4.10 ± 0.42	4.51 ± 0.58	5.34 ± 0.44[a,c]	5.76 ± 0.50[a,c]
Testosterone (ng/ml)	0.65 ± 0.15	0.31 ± 0.05[a]	1.27 ± 0.66	28.11 ± 4.06[b,d]	46.90 ± 17.9[b,d]
Estradiol (pg/ml)	40.0 ± 11.7	15.0 ± 2.0[a]	11.3 ± 1.3[a]	14.0 ± 4.0[a]	18.3 ± 4.1[a]

Data are mean ± SEM ($n = 5$ in each group). TP (0.01, 0.1, 1): Testosterone propionate (0.01, 0.1, 1 mg)
a; $P<0.05$, compared with intact control b; $P<0.01$, compared with intact control
c; $P<0.05$, compared with OVXTP (0) d; $P<0.01$, compared with OVXTP (0)

Table 2. The levels of total, unconjugated and conjugated BPA and the ratios of conjugated to total BPA in the supernatant after microsomal enzyme reaction

BPA	Intact control	OVXTP (0)	OVXTP (0.01)	OVXTP (0.1)	OVXTP (1)
Total (ng/ml)	10.53 ± 0.22	10.11 ± 0.20	10.29 ± 0.20	10.31 ± 0.05	10.60 ± 0.50
Unconjugated (ng/ml)	5.80 ± 0.61	5.85 ± 0.20	6.68 ± 0.82	7.02 ± 0.40	7.23 ± 0.57
Conjugated (ng/ml)	4.72 ± 0.57	4.26 ± 0.55	3.60 ± 0.95	3.30 ± 0.44	3.26 ± 0.26
Ratio of conjugated to total	0.448 ± 0.049	0.421 ± 0.045	0.350 ± 0.087	0.320 ± 0.041[a,b]	0.306 ± 0.031 [a,b]

Data are mean ± SEM (n = 5 in each group).
a; P<0.05, compared with intact control b; P<0.05, compared with OVX control

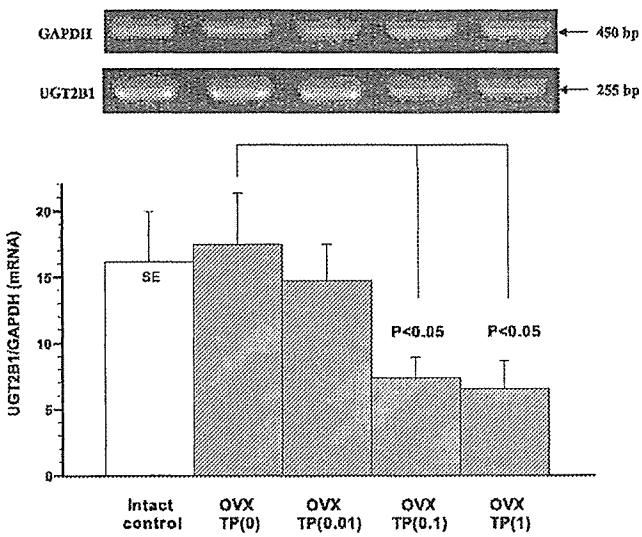

Fig. 3. Expression of UGT2B1 mRNA in the livers of female intact control, ovariectomized (OVX) control, and OVX rats treated with testosterone propionate (TP).
The relative expression level of UGT2B1 mRNA was significantly lower in the OVXTP (0.1) and OVXTP (1) groups compared with the OVXTP (0) group. Values are mean ± SEM (n = 5 in each group).

the OVXTP (0.1) and OVXTP (1) groups compared with the OVXTP (0) group (Fig. 3). The bands of GAPDH and UGT2B1 were detected as expected by electrophoretic analysis of the RT-PCR products, which were collected before the reaction plateau levels.

Discussion

BPA was detectable in all serum samples from rats. It was reported that BPA was found at a concentration of 0.6 ± 0.1 ng/ml in tap water in Korea [26]. We also detected 0.11–0.21 ng/ml of BPA in the tap water in our animal-breeding room (unpublished data). The main source of BPA consumed by the animals may be tap water, and the amount of BPA taken may be approximately 2–8 ng/day assuming that each rat drinks 20–40 ml of water. There was no difference in the volume of tap water drunk by the various groups of rats. Serum BPA concentrations in OVX rats with TP treatment showed a dose-dependent increase and were significantly higher at 0.1 and 1.0 mg of TP. The ratio of BPA glucuronidation in the OVX-rat liver microsome reaction was significantly lower in a TP dose-dependent manner. The clearance of BPA may be slowed in a TP dose-dependent manner, resulting in an increase of serum BPA concentrations with testosterone dose. Moreover, the relative expression level of UGT2B1 mRNA showed a TP dose-dependent decrease. These findings suggest that the amount of UGT2B1 enzyme catalyzing BPA glucuronidation is decreased by treatment with TP. To our knowledge, this is the first report of an effect of androgen on UGT2B1 enzyme.

In human beings, serum BPA concentrations are significantly higher in men than in women [16], and also in hyperandrogenemic women compared with normal women [17]. Moreover, we reported that there were significant positive correlations between serum BPA and total testosterone, free testosterone, androstenedione, and DHEAS concentrations in all female subjects [17]. Rat serum BPA concentrations are also significantly higher in males than in females, as in humans [23]. These *in vivo* data may implicate the relation of serum BPA concentrations with the androgen levels and are in accord with the present results.

Glucuronidation represents one of the conjugation reactions for the inactivation and excretion of both endobiotic and xenobiotic compounds. It is known that the metabolites of BPA include a monoglucuronide, a sulfate conjugate, and a glucuronide/sulfate diconjugate. Pritchett *et al.* [27] demonstrated that BPA-glucuronide was the major metabolite formed by hepatocytes of humans, rats and mice. The amount of BPA-sulfate was

much smaller (0–2%) than that of BPA-glucuronide, and especially, the former was not detected in female rats. The liver is a major site of glucuronidation as evidenced by the quantitative analysis. However, extrahepatic glucuronidation is also known to occur in organs such as the lung, kidney, gastrointestinal mucosa, prostate and olfactory epithelium [28–33]. The overall quantitative contribution of extrahepatic glucuronidation is much smaller compared with hepatic glucuronidation. Accordingly, glucuronidation in the liver influences the serum levels of both endobiotic and xenobiotic compounds. Extrahepatic glucuronidation might play an important role in the local regulation of the effects of compounds on various organs or tissues. In the present study, we investigated the conjugation of BPA in rat liver microsomes, and found that the conjugation rate of BPA was significantly lower under hyperandrogenemic conditions.

In conclusion, the results of enzyme analyses demonstrated that the ratio of BPA glucuronidation and the expression level of UGT2B1 mRNA were significantly lower under hyperandrogenemic conditions. The clearance of BPA is slowed concomitantly with the elevation of serum testosterone levels, resulting in an increase of serum BPA concentrations. The gender difference of serum BPA concentrations and the higher concentrations of BPA in hyperandrogenemic women may be due to the difference of androgenecity. The findings in this study stress the importance of the metabolism of endocrine disruptors.

Acknowledgements

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References

1. Sonnenschein C, Soto AM (1998) An updated review of environmental estrogen and androgen mimics and antagonists. *J Steroid Biochem Mol Biol* 65: 143–150.
2. Tsutsumi O (2005) Assessment of human contamination of estrogenic endocrine-disrupting chemicals and their risk for human reproduction. *J Steroid Biochem Mol Biol* 93: 325–330.
3. Feldman D (1997) Editorial: Estrogens from plastic — Are we being exposed? *Endocrinology* 138: 1777–1779.
4. Noda M, Komatsu H, Sano H (1999) HPLC analysis of dental resin composites components. *J Biomed Mater Res* 47: 374–378.
5. Krishnan AV, Stathis P, Permuth SF, Tokes L, Feldman D (1993) Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* 132: 2279–2286.
6. Olea N, Pulgar R, Perez P, Olea-Serrano F, Rivas A, Novillo-Fertrell A, Pedraza V, Soto AM, Sonnenschein C (1996) Estrogenicity of resin-based composites and sealants used in dentistry. *Environ Health Perspect* 104: 298–305.
7. Hiroi H, Tsutsumi O, Momoeda M, Takai Y, Osuga Y, Taketani Y (1999) Differential interactions of bisphenol A and 17β-estradiol with estrogen receptor α (ERα) and ERβ. *Endocr J* 46: 773–778.
8. Kurosawa T, Hiroi H, Tsutsumi O, Ishikawa T, Osuga Y, Fujiwara T, Inoue S, Muramatsu M, Momoeda M, Taketani Y (2002) The activity of bisphenol A depends on both the estrogen receptor subtype and cell type. *Endocr J* 49: 465–471.
9. Ashby J, Tinwell H (1998) Uterotrophic activity of bisphenol A in the immature rat. *Environ Health Perspect* 106: 719–720.
10. Von Saal F, Cooke PS, Buchanan DL, Palanza P, Thayer KA, Nage SC, Stetano P, Welshons WV (1998) A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. *Toxicol Indust Health* 14: 239–260.
11. Steinmetz R, Brown NG, Allen DL, Bigsby RM, BenJonathan N (1997) The environmental estrogen bisphenol A stimulates prolactin release *in vitro* and *in vivo*. *Endocrinology* 138: 1780–1786.
12. Takai Y, Tsutsumi O, Ikezuki Y, Hiroi H, Osuga Y, Momoeda M, Yano T, Taketani Y (2000) Estrogen receptor-mediated effects of a xenoestrogen, bisphenol A, on preimplantation mouse embryos. *Biochem Biophys Res Commun* 270: 918–921.
13. Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenbergh JG, vom Saal FS (1999) Exposure to bisphenol A advances puberty. *Nature* 401: 763–764.
14. Takai Y, Tsutsumi O, Ikezuki Y, Kamei Y, Osuga Y, Yano T, Taketani Y (2001) Preimplantation exposure to bisphenol A advances postnatal developement. *Reprod Toxicol* 15: 71–74.

15. Ikezuki Y, Tsutsumi O, Takai Y, Kamei Y, Taketani Y (2002) Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. *Hum Reprod* 17: 2839–2841.
16. Takeuchi T, Tsutsumi O (2002) Serum bisphenol A concentrations showed gender diffeerences, possibly linked to androgen levels. *Biochem Biophys Res Commun* 291: 76–78.
17. Takeuchi T, Tsutsumi O, Ikezuki Y, Takai Y, Taketani Y (2004) Positive relationship between androgen and the endocrine disruptor, bisphenol A, in normal women and women with ovarian dysfunction. *Endocr J* 51: 165–169.
18. Hiroi H, Tsutsumi O, Takeuchi T, Momoeda M, Ikezuki Y, Okamura A, Yokota H, Taketani Y (2004) Difference in serum bisphenol A concentrations in premenopausal normal women and women with endometrial hyperplasia. *Endocr J* 51: 595–600.
19. Mackenzie PI, Owens IS, Burchell B, *et al.* (1997) The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics* 7: 255–269.
20. Mackenzie PI (1986) Rat liver UDP-glucuronosyltransferase, sequence and expression of a cDNA encoding a phenobarbital-inducible form. *J Biol Chem* 261: 6119–6125.
21. Mackenzie PI, Rodbourn L (1990) Organization of the rat UDP glucuronosyltransferase UDPGT-2 gene and organization of its promoter. *J Biol Chem* 265: 11328–11332.
22. Yokota H, Iwano H, Endo M, Kobayashi T, Inoue H, Ikushiro S (1999) Glucuronidation of the environmental oestrogen bisphenol A by an isoform of UDP-glucuronosyltransferase, UGT2B1, in the rat liver. *Biochem J* 340: 405–409.
23. Takeuchi T, Tsutsumi O, Nakamura N, Ikezuki Y, Takai Y, Yano T, Taketani Y (2004) Gender difference in serum bisphenol A levels may be caused by liver UDP-glucuronosyltransferase activity in rats. *Biochem Biophys Res Commun* 325: 549–554.
24. Kodaira T, Kato I, Li J, Mochizuki T, Hoshino M, Usuki Y, Oguri H, Yanaihara N (2000) Novel ELISA for the measurement of immunoreactive bisphenol A. *Biomed Res* 21: 117–121.
25. Lowry OH, Rosebrough NJ, Farr AJ, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265–275.
26. Shin HS, Park CH, Park SJ, Pyo H (2001) Sensitive determination of bisphenol A in environmental water by gas chromatography with nitrogen-phosphorus detection after cyanomethylation. *J Chromatogr A* 912: 119–125.
27. Pritchett JJ, Kuester RK, Sipes IG (2002) Metabolism of bisphenol A in primary cultured hepatocytes from mice, rat, and humans. *Drug Metab Dispos* 30: 1180–1185.
28. Sipes IG, Gandolfi AJ (1991) Biotransformation of toxicants. In: Casarett LJ (ed) The Basic Science of Poisons. Pergamon Press, Oxford: 88–126.
29. Guillemette C, Lévesque E, Beaulieu M, Turgeon D, Hum DW, Bélanger A (1997) Differential regulation of two uridine diphospho-glucuronosyltransferase, UGT2B15 and UGT2B17, in human prostate LNCaP cells. *Endocrinology* 138: 2998–3005.
30. McGurk KA, Brierley CH, Burchell B (1998) Drug glucuronidation by human renal UDP-glucuronosyltransferase. *Biochem Pharmacol* 55: 1005–1012.
31. Burchell B (1991) Turning on and turning off the sense of smell. *Nature* 350: 16–17.
32. Lzard D, Zupko K, Poira Y, *et al.* (1991) Odorant signal termination by olfactory UDP-glucuronosyltransferase. *Nature* 349: 790–793.
33. Anders MW (1980) Metabolism of drugs by the kidney. *Kidney Int* 18: 636–647.

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Combined phospho-Akt and PTEN expressions associated with post-treatment hysterectomy after conservative progestin therapy in complex atypical hyperplasia and stage Ia, G1 adenocarcinoma of the endometrium

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Abstract

Young patients with complex atypical hyperplasia (CAH) or stage Ia, G1 adenocarcinoma (IaG1) of the endometrium, who desire to preserve fertility, can select the conservative therapy by oral progestin, medroxyprogesterone acetate (MPA). However, conservative treatments involve potential risks of progression and recurrence. In an attempt to find out molecular markers for sensitivity to MPA, we performed immunohistochemical analysis of PTEN, phospho-Akt, p53, ER and PgR in MPA-treated 31 cases with CAH or IaG1. Eleven of 12 cases (92%) with CAH and 15 of 19 cases (79%) with IaG1 demonstrated an initial complete response, while five patients underwent hysterectomy due to no response. Four of 11 responders (36%) with CAH and five of 15 responders (33%) with IaG1 later developed relapse. Five of nine patients (56%) with CAH and three of 11 patients (27%) with IaG1 became pregnant after infertility treatment. Immunohistochemical analysis revealed that phospho-Akt expression was significantly decreased by MPA administration ($p = 0.002$). Furthermore, combination of two factors, weak phosho-Akt or PTEN-null expression, was found to be significantly associated with receiving hysterectomy ($p = 0.04$), while each factor showed a trend without statistical significance ($p = 0.07$ and 0.2, respectively). Strong expression of both ER and PgR significantly correlated with successful pregnancy after infertility treatment following complete response to MPA ($p = 0.02$). Our observations in vivo suggest that anti-tumor action of MPA may be mediated by dephosphorylation of Akt, and that immunohistochemical evaluation of phospho-Akt and PTEN may be able to predict the outcome of MPA therapy.

Keywords: Akt; PTEN; MPA; Endometrial Carcinoma; Atypical hyperplasia

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1. Introduction

Young patients with complex atypical hyperplasia (CAH) or stage Ia, G1 adenocarcinoma (IaG1) of the endometrium, who wish to preserve their reproductive functions, can select the conservative therapy by oral progestin, medroxyprogesterone acetate (MPA) [1–3]. By progestin therapy, some patients are able to become free of disease and further conceive after infertility treatment, while others may have persistent disease and undergo hysterectomy. Although sensitivity to progestin is thought to be associated with presence of estrogen and progesterone receptors (ER, PgR), it is known that receptor-negative tumors can also respond to the agent, suggesting that there is another direct anti-tumor action of progestin without mediation by ER or PgR [4]. However, molecular mechanism of progestin's anti-tumor effect on endometrial tumors remains to be clarified yet.

PTEN, a tumor suppressor gene, is mutated in 30–55% of endometrial carcinomas [5–8] and also in about 20% of endometrial atypical hyperplasias, precancerous lesions of endometrial carcinoma [9,10]. PTEN acts as both a lipid and a protein phosphatase. The former activity is dephosphorylation of the 3-position of phosphatidylinositol 3,4,5-triphosphate, a second messenger of phosphatidylinositol 3-kinase (PI3K). PTEN antagonizes PI3K activity and negatively regulates the Akt-mediated signaling pathway, which is involved in cell survival and proliferation [11]. PTEN's protein phosphatase activity dephosphorylates focal adhesion kinase and inhibits cell migration, spreading and focal adhesion formation [11]. PTEN also regulates mitogen-activated protein kinase (MAPK) pathway through protein phosphatase activity [12]. It has been recently shown that estradiol-induced Akt phosphorylation is modulated by PTEN via MAPK pathway [13]. Moreover, Guzeloglu-Kayisli et al. have reported that estradiol may downregulate PTEN activity by increasing its phosphorylation, while progesterone is likely to regulate the PTEN pool by decreasing its phosphorylation and increasing its protein level [14]. These published findings suggest the possibility that anti-tumor effect of progestin may be mediated by upregulation of PTEN protein, subsequently leading to dephosphorylation of Akt.

The oncogene Akt is reported to be amplified in ovarian and pancreatic cancers [15,16]. This serine/threonine kinase is activated by phosphorylation, and mediates PI3K pathway through phosphorylating its substrates such as Bad, Caspase-9, FKHR, GSK3, Mdm2, p21 and p27, leading to diverse range of downstream effects including apoptosis, cell cycle progression and glycogen synthesis [17]. Although Akt amplification or mutation is not reported in endometrial carcinoma, a recent report has demonstrated a correlation between loss of PTEN expression and Akt phosphorylation in endometrial carcinoma [18], suggesting that genetic and epigenetic PTEN inactivation is leading to activation of PI3K/Akt pathway in endometrial carcinoma.

Tumor suppressor p53 is thought to be involved in chemosensitivity, as wild-type p53 is able to induce apoptotic signals in response to chemotherapeutic agents. It has been reported that p53 regulates transcription of PTEN [19]. Moreover, recent investigations have also revealed that PTEN inhibits degradation of p53 by recruiting Mdm-2 into the cytoplasm [20]. However, association of the expressions of p53 and PTEN with sensitivity to hormonal agents in endometrial tumor has not ever been examined.

In an attempt to find out molecular markers for sensitivity to MPA in young patients with endometrial tumors, we have evaluated expression levels of PTEN, phospho-Akt, p53, ER and PgR by immunohistochemistry in MPA-treated 31 cases with CAH or IaG1. Subsequently, we have compared our findings both prior to and during MPA therapy, and have correlated them with the outcomes of MPA and following infertility treatments. Our current observations in vivo provide novel implications for the conservative management of endometrial carcinoma and its precancerous lesions in young patients.

2. Materials and methods

2.1. Patients and samples

Paraffin-embedded specimens were collected from 31 patients with endometrial CAH or IaG1 who strongly wished to preserve fertility or could not receive surgery due to complications, and underwent MPA-treatment at the University of Tokyo Hospital between 1989 and 2003. All patients provided informed consent about the risk for recurrence of tumor and the research use of their samples. Before conservative treatment, histological examinations were performed by whole-wall endometrial curettage, and myometrial invasion and extra-uterine lesion were evaluated by transvaginal ultrasonography, magnetic resonance imaging (MRI) and/or computed

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tomography (CT). Histological classification and grading were conducted according to the World Health Organization criteria. Two patients who underwent hysterectomy due to the age over 40 were excluded from the analysis for MPA outcome, and 11 patients who did not attempt to conceive were excluded from the analysis for pregnancy. For comparing pre- and under-MPA sample pairs, 11 patients were excluded because of no tumor cells found in under-MPA samples due to complete response to the therapy, and one patient was excluded because of non-informative sample conditions. All pre- and under-MPA samplings were performed prior to the other therapies including estrogen plus progestin and infertility treatments.

2.2. Treatment

The patients were initially treated with MPA at 2.5–600 mg/day for 3–18 months (mostly 400–600 mg/day for 6 months, Table 1). The endometrium was curettaged and histologically evaluated for response to MPA at 2 or 3 months after initiation of the therapy. Endometrial sampling, either cytological or histological examination, was performed every 2–4 months after MPA therapy. The patients who did not respond to drugs or had recurrences of atypical hyperplasia at the age over 40 or had recurrences of adenocarcinoma at any age underwent hysterectomy. A complete response (CR) was defined as the absence of any carcinoma or atypical hyperplasia on follow-up endometrial curettings or hysterectomy after MPA therapy. A partial response (PR) was defined as a regression to complex or simple hyperplasia without atypia. At least 3 months of oral estrogen plus progestin (EP) was given after MPA therapy. Ovulation induction followed EP therapy for the patients wishing for pregnancy, while the remainder continued EP to prevent from unopposed estrogen and reduce the risk of relapses. The median follow-up period was 40.7 months, ranging from 2 to 109 months, after the beginning of the conservative therapy at our hospital.

2.3. Immunohistochemistry

A 4 μm section was cut from the paraffin blocks of CAH and G1 adenocarcinoma. Each section was mounted on a silane-coated glass slide, deparaffinized, and soaked for 15 min at room temperature in 0.3% H_2O_2/methanol to block endogenous peroxidase. Sections were incubated with antibodies against PTEN (mouse monoclonal, PTEN A2B1 [1:50 dilution]; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Akt (rabbit polyclonal, Anti-phospho-Akt1/PKBα (Ser473) [1:50 dilution]; Upstate, Waltham, MA), p53 (mouse monoclonal, DO-7 [1:200 dilution]; Novocastra, Newcastle, UK), ER (mouse monoclonal, 1D5 [1:200 dilution]; DAKO, Glostrup, Denmark), and PgR (mouse monoclonal, pgR636 [1:200 dilution]; DAKO) overnight at 4 °C. The primary antibody was visualized using the Histofine Simple Stain PO(M) kit (Nichirei, Tokyo, Japan) according to the instruction manual. The slide was counterstained with hematoxylin. Normal endometrial epithelium provided an internal positive control, and negative controls without addition of primary antibody showed low background staining in all cases. Blinded for patient characteristics and outcome, the intensity of staining for PTEN, phospho-Akt, p53, ER and PgR in tumor cells was scored (0–3) by two investigators (TM and YT) independently as follows: the group assigned 3 showed increased staining intensity; the group assigned 2 showed equal staining intensity compared to the corresponding normal endometrial epithelium; the group assigned 1 had decreased staining intensity; and the group assigned 0 had no trace of staining. If the staining pattern in tumor cells was heterogeneous, the score was recorded such as "0 + 1", meaning that tumor cells with negative and decreased staining are mixed.

2.4. Statistical analyses

In order to elucidate factors associated with responses to MPA or infertility treatment, the immunohistochemical findings were correlated with undergoing hysterectomy or successful pregnancy using χ^2 test or Fisher's exact test, where appropriate. Those findings were also compared prior to and during MPA treatment using Wilcoxon signed-ranks test. p values less than 0.05 were considered statistically significant.

3. Results

3.1. Patients and treatment outcome

Thirty-one patients with CAH or IaG1 of the endometrium received conservative MPA therapy (Table 1). The patient age ranged from 19 to 60 years (average, 32.3). This study included one patient of age over 40 (60 years old, case 11) who had to receive conservative therapy due to complications.

Twelve patients with CAH were initially treated with MPA at 2.5–600 mg/day for 4–18 months. Eleven of 12 patients (92%) demonstrated an initial CR to MPA, but one patient (8%) underwent hysterectomy due to not responding to MPA. Relapses were observed in 4 among 11 patients (36%) who had shown initial CR. Two patients selected MPA therapy again and became free of disease, and the other two received hysterectomy due to age of over 40. Nine patients attempted to become pregnant, and five women (56%) became pregnant after infertility treatment. Two women successfully delivered two healthy and full-term infants, and one woman is still pregnant with singleton. All patients but one were alive without evidence of disease at the last follow-up. Case 11 of age 60 showed CR and had no relapse, but died of liver cirrhosis.

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Table 1
Clinicopathological findings and follow-up of 31 patients with CAH or IaGi treated with MPA

Case	Age	G/P[a]	Histology	MPA Therapy		Relapse	Pregnancy	Hysterectomy	Other therapy	Current status (months)
				Dose (mg/day) × Duration(months)	Response					
1	31	0/0	CAH	600 × 6,600 × 6[b]	CR[c]	Yes	...	No	EP[d], Clomid, Sexovid, IVF/ET[e]	NED[f] (37)
2	35	1/1	CAH	600 × 6	CR	No	Abortion	No	EP, Clomid, Drilling, IVF/ET	NED(56)
3	34	0/0	CAH	600 × 6	CR	No	Singleton	No	EP, IVF/ET	NED(13)
4	37	0/0	CAH	600 × 6	CR	Yes	...	Yes (age ⩾ 40)[g]	EP	NED(42)
5	33	0/0	CAH	600 × 6	CR	No	...	No	EP, Clomid, IVF/ET	NED(12)
6	28	0/0	CAH	600 × 4	CR	No	...	No	EP, HMG/HCG, Drilling, Clomid, IVF/ET	NED(49)
7	32	0/0	CAH	400 × 7.5, 400 × 6[b]	CR	Yes	CPB[h]	No	EP, Clomid, Drilling, IVF/ET	NED(92)
8	33	1/0	CAH	400 × 6	NC[i]	No	...	Yes	EP	NED(69)
9	37	0/0	CAH	400 × 6	CR	Yes	Abortion × 2	Yes (age ⩾ 40)[g]	EP, Clomid	NED(41)
10	28	0/0	CAH	400 × 6	CR	No	Abortion, NFTD[j]	No	EP, Clomid, IVF/ET	NED(70)
11	60	3/3	CAH	2.5-20 × 14	CR	No	...	No	...	DOC[k] (15)
12	34	0/0	CAH	15 × 18	CR	No	...	No	EP, IVF/ET	NED(23)
13	28	0/0	GI	600 × 6, 600 × 6[b]	CR	Yes(AH)[j]	...	No	EP, IVF/ET	NED(30)
14	37	0/0	GI	600 × 6, 600 × 6[b]	CR	Yes(AH)[j]	...	No	EP, Clomid, IVF/ET	NED(43)
15	32	0/0	GI	600 × 6	CR	No	...	No	EP, Clomid, IVF/ET	NED(26)
16	33	0/0	GI	600 × 6	CR	No	Twin	No	EP, IVF/ET	NED(32)
17	35	0/0	GI	600 × 6	CR	No	Singleton	No	EP, IVF/ET	NED(27)
18	27	0/0	GI	600 × 6	CR	No	...	No	EP	NED(35)
19	36	0/0	GI	600 × 6	CR	Yes	...	Yes	EP, Clomid	NED(51)
20	33	0/0	GI	600 × 6	CR	No	...	No	EP, IVF/ET	NED(17)
21	32	0/0	GI	600 × 6	CR	No	...	No	EP, IVF/ET	NED(17)
22	26	0/0	GI	600 × 6	CR	Yes	NFTD	Yes	EP, Clomid, CAP[m], WP[n]	NED(59)
23	20	0/0	GI	600 × 6	CR	No	...	No	EP, Clomid	NED(51)
24	35	0/0	GI	600 × 4	NC	No	...	Yes	...	NED(49)
25	30	0/0	GI	600 × 4	NC	No	...	Yes	...	NED(20)
26	32	0/0	GI	600 × 4	NC	No	...	Yes	...	NED(18)
27	19	0/0	GI	400 × 8	CR	No	...	No	EP	NED(102)
28	24	0/0	GI	400 × 4, 600 × 4	CR	No	...	No	EP, IVF/ET	NED(70)
29	34	0/0	GI	400 × 2, 600 × 4	CR	Yes	...	Yes	EP	NED(99)
30	29	0/0	GI	400 × 2, 600 × 1	PD[o]	No	...	Yes	...	NED(109)
31	36	0/0	GI	400 × 2	CR	No	...	No	...	NED(2)

[a] Gravidity/Parity.
[b] Secondary MPA therapy for 6 months was performed for relapse of atypical hyperplasia.
[c] Complete response.
[d] Oral estrogen plus progestin.
[e] In vitro fertilization and embryo transfer.
[f] No evidence of disease.
[g] Hysterectomy performed due to age over 40.
[h] Cesarean section.
[i] No change.
[j] Normal full-term delivery.
[k] Died of other cause.
[l] Relapse of atypical hyperplasia.
[m] Cyclophosphamide, Doxorubicin, and Cisplatin.
[n] Whole-pelvis irradiation.
[o] Progressive disease.

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Nineteen patients with IaG1 were initially treated with MPA at 400–600 mg/day for 3–8 months. Fifteen of 19 patients (79%) demonstrated an initial CR to MPA, but four patients (21%) did not respond and underwent hysterectomy. Relapses were observed in five among 15 patients (33%) who had shown initial CR. Two patients with relapses of atypical hyperplasia selected MPA therapy again and became free of disease, and the other three patients with relapses of carcinoma received hysterectomy. Eleven patients attempted to become pregnant, and three women (27%) became pregnant after infertility treatment. One woman successfully delivered one healthy and full-term infant, and the other two women are still pregnant (singleton and twin). All patients were alive without evidence of disease at the last follow-up.

3.2. Phospho-Akt/PTEN expressions and MPA outcome

In our immunohistochemical analysis of 31 endometrial tumors, PTEN expression was detected mainly in cytoplasm of endometrial glands (Fig. 1A). P53, ER and PgR expressions were observed in nucleus (Fig. 1E, G and H), and phospho-Akt expression was observed both in cytoplasm and nucleus (Fig. 1C and D). Because cytoplasmic Akt appears to be critical for its oncogenic function according to recent studies [21,22], and the function of nuclear Akt has not been fully clarified, we applied cytoplasmic expressions of phospho-Akt in tumors for statistical evaluation. Although we also examined presence or absence of nuclear PTEN expression in tumors, no association with outcome or administration of MPA was observed (data not shown). Table 2 summarizes the immunohistochemical evaluations of pre- and under-MPA samples from the 31 cases with endometrial tumors. Presence of PTEN-null tumor cells (IHC score including 0) in pre-MPA samples was observed in three of 12 (25%) cases with CAH and nine of 19 (47%) cases with IaG1. Strong phospho-Akt expression (IHC score $\geqslant$ 2) in pre-MPA samples was observed in nine out of 12 (75%) cases with CAH and 13 out of 19 (68%) cases with IaG1. Strong expressions of p53 (IHC score $\geqslant$ 2) in pre-MPA samples were observed in two of 12 (17%) cases with CAH and six of 19 (32%) cases with IaG1. In order to examine the correlation between MPA outcome and the expression profile in tumors, we compared the proportion of patients who received hysterectomy according to different expression levels. Patients who underwent hysterectomy due to age over 40 were excluded from the analysis. Five of 12 cases (42%) with PTEN-null tumor cells and three of 17 cases (18%) without PTEN-null tumor cells in pre-MPA samples received hysterectomy due to relapse or no response to MPA. Three of 20 cases (15%) with strong phospho-Akt expression (IHC score $\geqslant$ 2) and five of nine cases (56%) with weak phospho-Akt expression (IHC score $\leqslant$ 1) in pre-MPA samples resulted in hysterectomy. Presence of PTEN-null tumor cells (IHC score including 0) and weak phospho-Akt expression (IHC score $\leqslant$ 1) in pre-MPA samples were both found to be associated with a trend towards receiving hysterectomy without statistical significance ($p = 0.2$ and 0.07, respectively by Fisher's exact test; Table 3). Combining these two factors, however, PTEN-null or weak phosho-Akt expression was found to be significantly associated with receiving hysterectomy ($p = 0.04$ by Fisher's exact test; Table 3). Because these two factors, PTEN null and weak phospho-Akt, are contradictory in terms of the signaling pathway, we examined whether the cells exhibiting both of the staining patterns are the same or a mix of different cells in the samples. Interestingly, the two staining patterns were found to mostly coexist in the same tumor cells (Fig. 1I and J). No association was found between expression of p53, ER or PgR and undergoing hysterectomy. Histology of G1 showed association with tendency to receive hysterectomy, but did not reach statistical significance (Table 3). Although we also correlated these factors with initial response to MPA or relapse after MPA, no association was observed (data not shown).

3.3. Steroid receptors expressions and pregnancy after infertility treatment

Subsequently, we examined association between expressions of steroid receptors in tumors and successful pregnancy after infertility treatment following CR to MPA. Patients who did not attempt to conceive were excluded from the statistical analysis. Strong expression of both ER (IHC score $\geqslant$ 2) and PgR (IHC score = 3) in tumor cells in pre-MPA samples was found to be significantly associated with successful pregnancy ($p = 0.02$ by Fisher's exact test; Table 3). Although each of strong ER expression (IHC score $\geqslant$ 2) or strong PgR expression (IHC score = 3) was associated with a trend towards successful pregnancy, they were not statistically significant ($p = 0.06$ and 0.2, respectively, by Fisher's exact test; Table 3).

3.4. Comparison of immunohistochemical findings in pre- and under-MPA sample pairs

Finally, we compared the expressions of the genes in pre- and under-MPA treatment sample pairs. By MPA treatment, phospho-Akt expression was significantly decreased and ER expression was significantly increased ($p = 0.002$ and 0.002, respectively, by Wilcoxon signed-ranks test, Figs. 1C, D and 2). No difference was observed in PTEN, p53 or PgR expressions (Fig. 2).

4. Discussion

PTEN mutation is highly associated with endometrial endometrioid adenocarcinoma, which is thought to be estrogen-dependent type of tumor

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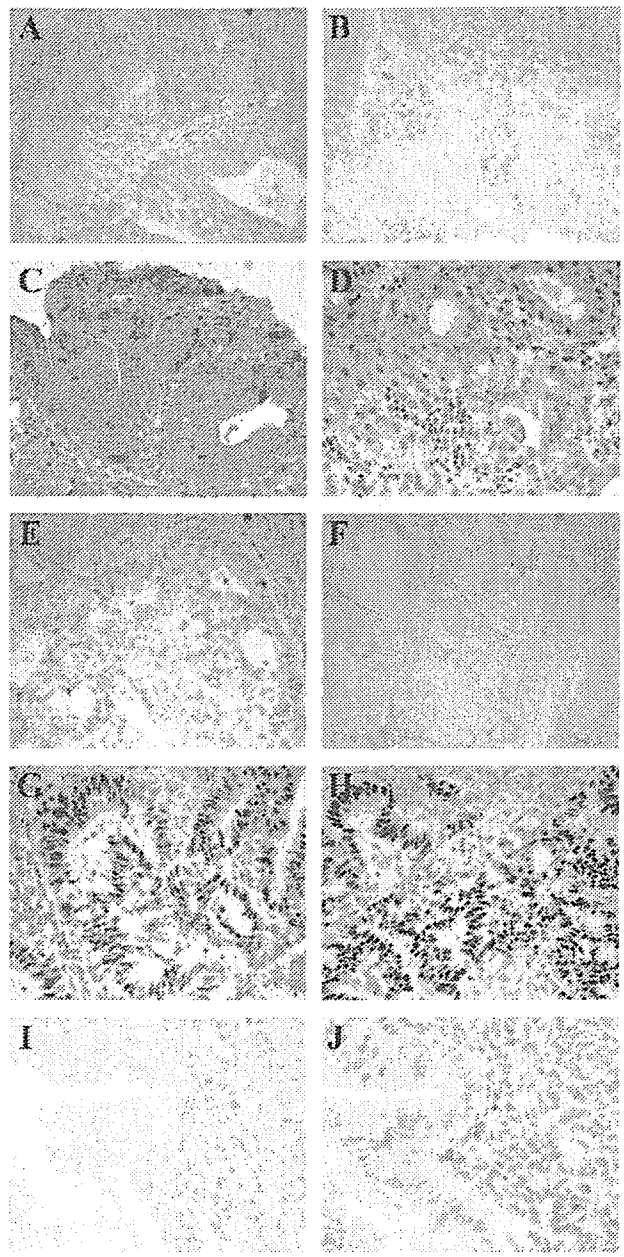

Fig. 1. Immunohistochemical staining patterns of PTEN, p-Akt, p53, ER and PgR in endometrial tumors. (A) Positive staining for PTEN in CAH (IHC score = 3). (B) Negative staining for PTEN in CAH (IHC score = 0). (C) Strong staining of p-Akt in pre-MPA sample of IaG1 (IHC score = 3). (D) Decreased staining of p-Akt in under-MPA sample of IaG1 (IHC score = 1). (E) Positive staining for p53 in IaG1 (IHC score = 1 + 2). (F) Negative staining for p53 in IaG1 (IHC score = 0). (G) Positive staining for ER in IaG1 (IHC score = 3). (H) Positive staining for PgR in IaG1 (IHC score = 3). (I) PTEN-null cells in CAH (IHC score = 0). (J) Low phospho-Akt cells in CAH (IHC score = 1). (C) and (D) are samples from the same patient. (I) and (J) are different stainings of the same sample. (A–H) ×40; (I)–(J) ×60.

[7]. Endogenous or exogenous estrogen unopposed with progesterone is thought to contribute to the pathogenesis of endometrial tumor of this hystotype [4]. Recently, ERα has been reported to bind in a ligand-dependent manner to p85α, the regulatory subunit of PI3K, leading to activation of Akt [23]. Therefore, loss of PTEN function can be thought to promote the signal transduction of estrogen/PI3K/Akt pathway in tumors. However, the mechanism of anti-tumor action by progestin to

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