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Neonatal Exposure to p-tert-octylphenol Causes Abnormal Expression of Estrogen Receptor α and Subsequent Alteration of Cell Proliferating Activity in the Developing Donryu Rat Uterus

MIDORI YOSHIDA, AKIKO TAKENAKA, SHIN-ICHI KATSUDA, YUJI KUROKAWA, AND AKIHIKO MAEKAWA

Department of Pathology, Sasaki Institute, Chiyoka-ku, Tokyo 101-0062, Japan

ABSTRACT

In the present study, we investigated immunohistochemically the time-course alterations in estrogen receptor α (ER) expression and cell proliferating activity in the developing uteri of Donryu rats exposed neonatally to a high dose p-tert-octylphenol (OP), an endocrine disrupting chemical (EDC). OP-treatment (sc injections of 100 mg/kg, every other day from postnatal days 1 to 15) induced an early and enhanced ER expression in the luminal epithelium compared with age-matched controls from postnatal day (PND) 10, and increased proliferating cell nuclear antigen (PCNA) positive cells up to PND21. At PND28, ER expression in the luminal epithelium of the OP-treated group was decreased, in association with decline in the luminal epithelial areas. PND14, the second week of life, is coincident with the normal time for differentiation when the luminal epithelium invaginates into the stroma to form uterine glands. OP-treatment, however, delayed and inhibited gland-formation, and suppressed ER expression in the invaginated-luminal and glandular epithelium at this time. These results indicate that ER expression in these sites is strongly linked with cell proliferating activity. In stromal cells, ER was expressed from PND6 in both groups without any PCNA positive cells, but significantly lower values were noted in the OP-treated group up to PND10. Our immunohistochemical investigation did not reveal any abnormalities in expression of the proto-oncogene c-fos, mitotic inhibitor p21, or epidermal growth factor antigen, although the apoptotic index in the luminal epithelium was slightly increased in the OP-treated group. These results demonstrate neonatal effects of a high dose of OP, already detectable at PND10, with early and enhanced ER expression, resulting in increase of cell proliferative activity in the luminal epithelium, though expression in the glandular epithelium was suppressed in relation to inhibited gland-genesis. The present study thus suggests that neonatal exposure to high doses of EDCs with estrogenic activity can induce abnormal differentiation in the developing rat uteri via abnormal ER expression and subsequent alteration of cell proliferating activity.

Keywords. Estrogen receptor α; PCNA; uterus; neonatal exposure; p-tert-octylphenol; rat.

INTRODUCTION

The developing rodent uterus undergoes a period of rapid growth and differentiation during the first 2 weeks of postnatal life. During this period, luminal epithelial cells invaginate into the underlying stroma to form uterine glands (14). In the rat, the uterine growth phase in this period coincides with an elevation of serum estradiol levels beginning on postnatal day (PND) 9 and therefore the roles of endogenous estrogen and its receptor in uterine growth and differentiation are of considerable interest (13). Because effects of estrogens are mostly mediated via binding to estrogen receptor α (ER), it is important to determine the ontogeny of ER in the developing uterus taking into account the fact that uterine responses to estrogen stimulation are different among the constituent cell types (14, 16, 17, 37).

Endogenous estrogen is important for normal reproductive tract development, and inappropriate estrogen exposures can cause serious and irreversible effects on target organs such as the uterus and vagina, in association with abnormal development of the hypothalamus resulting in hypothalamo-pituitary-gonadal disorders (4, 18, 22, 31). The most striking examples in humans are vaginal adenocarcinoma and uterine hypoplasia associated with fetal diethylstilbestrol (DES) exposure (19). In rats, the uterus at birth corresponds developmentally to the fetal uterus at gestation day 100 in human beings (20), and treatment with estrogens such as DES to neonatal rats or mice leads not only to profound uterine hypoplasia but also adenocarcinomas in the uterus and/or the vagina, thus providing useful models for the human situation (1, 9, 28–30, 34). Additionally, DES and other estrogen/antiestrogens can inhibit uterine gland-genesis in the rat and mouse, so that developmental exposure to exogenous estrogens leads to irreversible abnormalities of both growth and differentiation (4, 7, 8).

Recently, environmental pollution with man-made chemicals having weak estrogenic effects, which may disturb the endocrine systems of wildlife and human beings, has become an important social problem (10). In particular, effects of perinatal exposure to such endocrine disrupting chemicals (EDCs) are a focus of attention, because of their potential to act as estrogens and influence the growth and differentiation of organs. Alkylphenols such as nonylphenol and octylphenol are derived from biodegradation of alkylphenol ethoxylates, nonionic surfactants that are widely used as detergents in industry (15). It is well known that these alkylphenols exert weak estrogenic activity in vitro and in vivo, resulting from binding to ER of mammalian cells (11, 21, 36, 38). In our previous studies, the effects of neonatal exposure to high dose p-tert-octylphenol (OP) on the reproductive organs of male and female Donryu rats were in line with those of estrogens, particularly long-term persistent irreversible effects

Address correspondence to: Midori Yoshida, Department of Pathology, Sasaki Institute, 2-2 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062 Japan; e-mail: pathol@sasaki.or.jp.

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in females: lower gonadotropin levels at prepuberty, inhibition of uterine gland-genesis, persistent estrus as evidenced by vaginal cytology, and polycystic ovaries (18, 22, 39). At 8 weeks of age, treated females exhibited luminal epithelial hyperplasias with overexpression of ER mRNA, similar to preneoplastic lesions for uterine adenocarcinomas in this rat strain (22, 27). It has been known since the 1980s that neonatal exposure to inappropriate estrogen can cause adenocarcinomas in the uterus or vagina of humans or rodents but the pathogenesis linking abnormal uterine development to adenocarcinomas remains to be clarified. In addition, the mechanism whereby neonatal exposure to estrogens causes abnormal uterine development has not yet to be detailed, although stimulation of certain signaling pathways involving growth factors and cell cycle-related genes is presumably important as cues of the mitogenic effect of 17 β-estradiol (E2) on the uteri (24, 41, 42).

In the present study, the time-course alterations in ER expression and cell proliferating activity of different cell types in the endometrium of developing uteri of rats neonatally exposed to a high dose of OP were investigated using an immunohistochemical approach. Growth factors or proteins controlling cell cycles were also investigated as other factors related to ER expression. In the present study, we used the Donryu rats because this strain rat was documented to show high incidence for spontaneous development of endometrial adenocarcinomas associated with age-related hormonal imbalance (27).

METHODS

Animals: Pregnant female Crj:Donryu rats were purchased from Charles River Japan Inc (Kanagawa, Japan). The day of birth was designated as postnatal day (PND) 0. Litter size was adjusted to about 10 pups/dam at PND 6, and all pups were weaned at PND 21 and females were separated from males. A total of 52 female pups was investigated in this study. The rats were housed 4 animals per plastic cage, and they were kept in an air-conditioned animal room under constant conditions of 24 ± 2°C and 55 ± 10% humidity with a 12-hour light/dark cycle and maintained on basal diet, CRF-1 (Oriental Yeast Inc, Tokyo, Japan) and tap water ad libitum. Animal care and use followed the NIH Guide for the Care and Use of Laboratory Animals.

Treatment: Based on our previous studies (21, 22, 38, 39), we selected sc treatment with 100 mg/kg OP (Wako Pure Chemical, Osaka) as effective for estrogenic activity on the female reproductive tract in rats when exposed neonatally and postnatally, although this dose level is high relative to what exists in the human environment (5). Animals were administered OP at doses of 0 (control) and 100 mg/kg at every other day from PND 1 (within 24 hours after birth) until PND 15. The repeated injections of OP did not affect the growth curve or clinical observations except for slight scar formation at the injection sites, observed throughout the treatment period.

Pathology: Four or five animals per group were euthanized for histopathological examination at PNDs 6, 10, 14, 21, and 28. To eliminate potential litter effects, pups for necropsy were selected from different dams at each time point. After necropsy the uteri were fixed in 10% neutral-buffered formaldehyde solution and cross-sections from the upper, middle, and lower portions of both uterine horns were routinely processed to paraffin-embedded sections for hematoxylin-eosin (H-E) staining.

Immunohistochemistry: Serial sections cut at 4 μm were mounted on coated glass slides and stained immunohistochemically with the antibodies listed in Table 1: estrogen receptor α (ER) (Dako Japan, Kyoto), proliferating cell nuclear antigen (PCNA) (Dako Japan), p21 protein (Santa Cruz Biotechnology Inc, CA), c-fos (Santa Cruz) and epidermal growth factor (EGF) (Biomedical Technologies Inc, MA). Immunoreactive complexes were detected by the avidin-biotin complex method, LSAB kit (Dako Japan) for polyclonal antibodies, or the polymer immuno-complex method, Ensivion$^+$ (Dako Japan) for mouse monoclonal antibodies, and visualized with 3,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemical) as the chromogen. To examine in situ analysis for DNA fragmentation in the uterus, a kit for detection of terminal deoxynucleotidyl tranferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) (Wako Pure Chemical) was employed.

Morphometric Analysis of ER and Proliferating Cell Nuclear Antigen (PCNA) Labeling Indices: To investigate the relationship between ER expression and cell proliferating activity in the endometrium, a morphometric analysis was performed using an IPAP image analyzing system (Sumika Technoservice Co, Osaka) as described previously (35). The endometrium of the uterine horn was histologically classified into four cell types: luminal epithelium, invaginated-luminal epithelium, or glandular epithelium, and stromal cells. Invaginated-luminal epithelium is recognized as early glandular epithelium. Labeling indices for ER and PCNA were analyzed using the following approach: For each epithelial cell type, the positive nuclear area per total nuclear area of several cross-sections (μm2) was measured using the IPAP. For stromal cells, the ratio of the positive to total nuclear area was also calculated in the same manner.

Statistical Analysis: Data were expressed as mean values with the standard deviation (SD). Differences in the data for ER and PCNA between control and treated animals were evaluated for statistical significance using the Student's

TABLE 1.—Antibodies for immunohistochemistry.

Antibodies	Primary antibodies	Dilution	Source
Estrogen receptora α (ER)	Monoclonal IgG1(1D5) against human ER 67-kDa protein	1:50	Dako Japan, Kyoto
Proliferating cell nuclear antigen (PCNA)	Monoclonal IgG2(PC10) against rat PCNA 36kDa protein	1:100	Dako Japan, Kyoto
Epidermal growth factor (EGF)	Polyclonal rabbit IgG(BT-216) against rat EGF protein	1:100	Biomedical Technologies Inc, MA
p21 protein	Monoclonal mouse IgG2b(sc-6246) against full length p21protein of mouse origin	1:50	Santa Cruz Biotechnology Inc, CA
c-Fos protein	Polyclonal rabbit IgG against 62-kDa c-fos protein	1:100	Santa Cruz Biotechnology Inc, CA

t-test. The comparison was considered statistically significant if the *p* value was less than 0.05.

RESULTS

In the uteri stained by H-E, no morphological abnormalities were detectable in OP-treated animals except for inhibition of gland-genesis after PND14. Total cell areas as well as cell height of the luminal epithelium in OP-treated group were slightly but significantly increased at PND14 (Figure 1), but the former was significantly depressed at PND28 compared with that of the controls (Figure 1).

Expression of the 2 proteins, ER and PCNA, showed distinct differences among the luminal, invaginated-luminal and glandular epithelial cells and stromal cells, as shown in Figures 2–4. Positive areas for each cell type are shown in Figure 4.

ER (Figures 2 and 4): Immunodetectable ER was assessed in nuclei of luminal and glandular epithelial cells, and stromal cells of the uterus in OP-treated and control rats. In luminal epithelial cells, ER was not detected at PND 6 in either group (Figure 2a, b). At PND10, slightly positive areas of ER began to appear in luminal epithelial cells in the OP-treated, but not the control animals (Figure 2c, d). At PND 14, ER expression in luminal epithelial cells was sparse in the controls, but increased in OP-treated rats (Figure 2e, f). At this time, elements of invaginated-luminal epithelium within the stroma, the first step of gland-genesis, began to be recognized, associated with intense expression of ER in the controls. OP-treatment significantly inhibited this uterine gland-genesis. At PND21, ER expression in luminal epithelial cells was more abundant and diffuse in OP-treated rats (Figure 2g, h). ER-expressing areas of glandular epithelium of the OP-treated group were depressed at PND 21 (Figure 4), but the intensity of expression in fully developed glands was not different between the groups (Figure 2g, h). At PND28, ER-positive areas rapidly decreased in the OP-treated group in line with the reduction in total area of the luminal epithelium (Figures 1 and 4).

Interestingly, intense ER expression in stromal cells was evident at PND 6 in both groups, the expression being slightly but significantly suppressed in the OP-treated group up to PND10, compared with age-matched controls (Figures 2 and 4). After PND14, ER was consistently expressed in stromal cells of both groups without significant differences up to PND28.

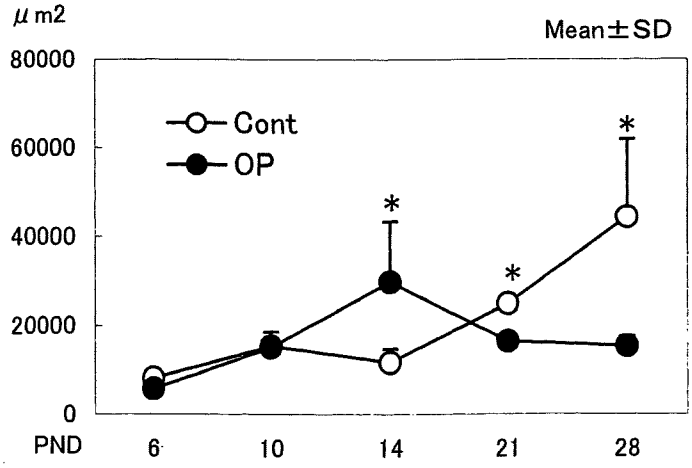

FIGURE 1.—Total cell areas of luminal epithelium. Open and closed circles are the control and OP-treated groups, respectively. $*p < 0.05$.

PCNA (Figures 3 and 4): PCNA positive cells could not be detected in any cell type in the endometrium at PND6. At PND10 scattered positive luminal epithelial and stromal cells were evident in the OP-treated group but not the control group (Figure 3a, b). At PND14, PCNA positive cells in the luminal epithelium of the OP-treated group were also increased as compared with the controls. At this time, PCNA-positive invaginated luminal or glandular epithelial cell nuclei began to increase with gland-genesis in the control group compared to the OP-treated group (Figure 3c, d). At PND21, PCNA positive cells in the luminal epithelium in the OP-treated group showed a similar tendency to those in the control group at PND 14 (Figure 3e, f). At PND28, no differences were evident for each epithelial cell component between the groups (Figure 3g, h). PCNA-positive stromal cells were present at low levels in both groups up to PND21, but they were significantly increased in the OP-treated as compared to the control group at PND28.

Apoptosis (Figure 5): Throughout the observation period, a few apoptotic cells were scattered in the luminal or glandular epithelium in both groups. They were relatively more frequent in the OP-treated luminal epithelium until PND14. After this time point, there were no differences between the OP-treated and control groups.

p21, c-fos, EGF: Immunohistochemical examination did not reveal any expression of p21 and c-fos in the uteri of either group. Additionally no differences were apparent in expression of EGF between OP-treated and control animals at any of the time points examined.

DISCUSSION

It is well known that neonatal exposure to compounds with estrogenic activity causes serious and irreversible effects on target organs such as the uterus and vagina. As estrogenic effects are mediated mainly via ER, it is important to define the levels of ER in the developing uteri of rats exposed to estrogens including EDCs. The uterine endometrium is composed of luminal and glandular epithelial cells and stromal cells. The present study demonstrated that an immunohistochemical approach allows recognition of abnormal patterns of ER and PCNA positive cells of each cell type in the developing uteri of rats exposed neonatally to a high dose of OP.

Neonatal exposure to OP induced early and enhanced ER expression and increased PCNA positive expression in the luminal epithelium of treated animals up to PND21. At PND28, ER expression in the luminal epithelium was decreased in the OP-treated group but this appeared to be related to depression of the total area of luminal epithelium at this time. Sato et al (32) also reported early ER expression in the luminal epithelium of uteri induced by neonatal exposure to estrogens/anti-estrogens in mice, whereas other investigators indicated that cytosolic estrogen receptors were decreased in mice neonatally exposed to DES (6). In other studies ER expression in the uterine epithelium of control rats began to

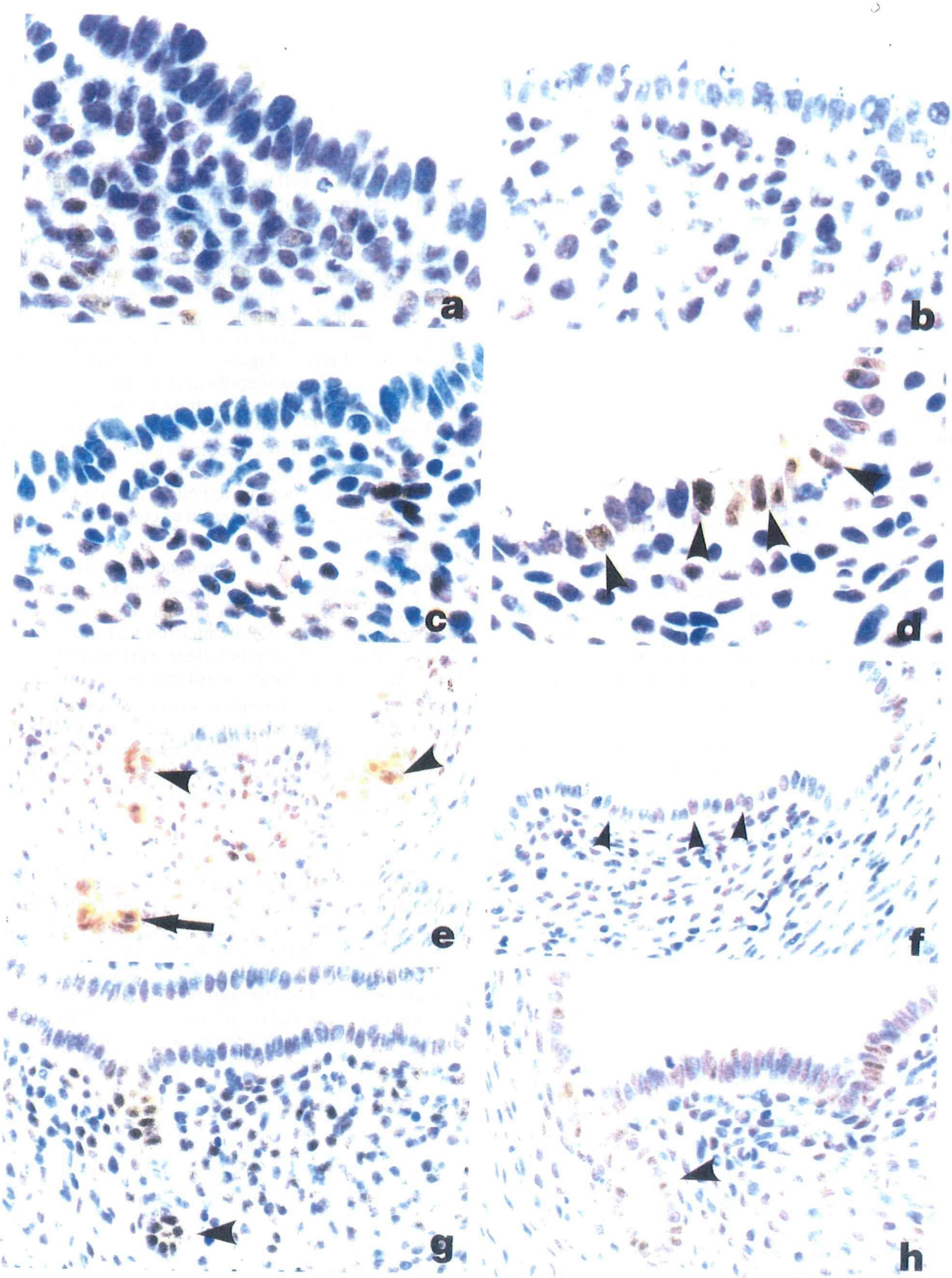

FIGURE 2.—Immunohistochemical staining of ER expression in the uterine endometrium at PND6 (a, b), PND10 (c, d), PND14 (e, f) and PND21(g, h). a, c, e, g: the control group. b, d, f, h: the OP-treated group. ER expression in the luminal epithelium was not detected at PND6 in either group (a, b) and PND10 in the control group (c), but could be recognized after OP treatment at PND10 in the OP-treated group (arrowheads) (d). At PNDs 6 and 10, ER expression in stromal cells of OP-treated animals was lower than in controls. At PND14, ER expression in the invaginated luminal (arrowheads) and glandular epithelium (arrow) was observed in the control group with gland-genesis (e), which was inhibited in the OP-treated group (f). In the luminal epithelium, ER expression was increased in the OP-treated group (arrowheads) (f). At PND21, ER expression in the luminal epithelium was greater in the OP-treated (h) than the control group (g), while intensity of the expression in the completed glands was comparable (arrowheads). a–d: ×200. e–h: ×100.

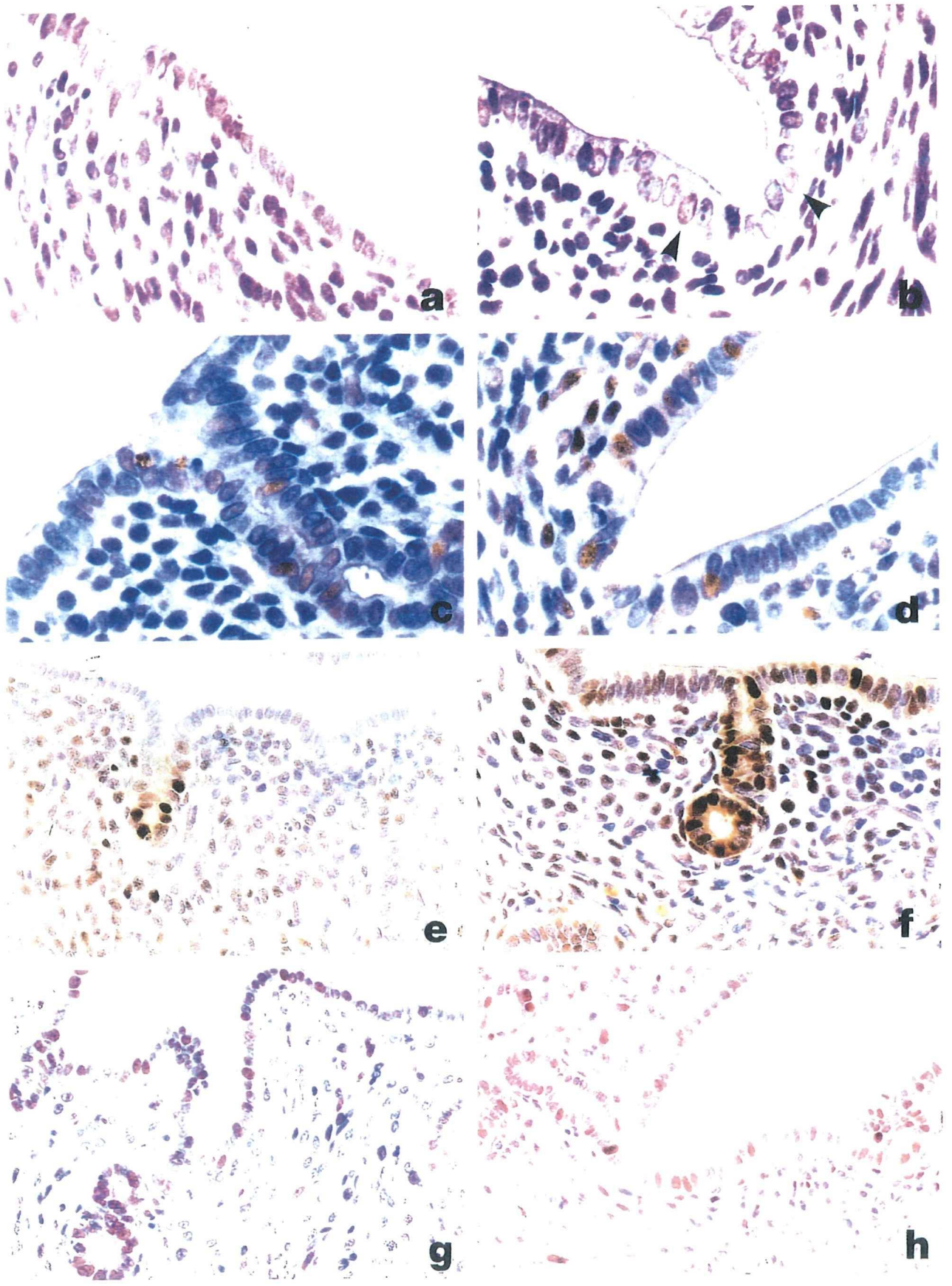

FIGURE 3.—Immunohistochemical staining of PCNA in the endometrium at PND10 (a, b), PND14 (c, d), PND21 (e, f) and PND28 (g, h). a, c, e, g: the control group. b, d, f, h: the OP-treated group. At PND10, PNCA positive cells began to appear in the luminal epithelium (arrowheads) of the OP-treated group. At PNDs 14 and 21, PCNA positive cells in the luminal epithlium were increased in the OP-treated animals (d, f), compared with controls (c, e). At PND 28, PCNA positive cells were observed in both groups at similar frequencies (g, h). a–d: ×200, e–h: ×100.

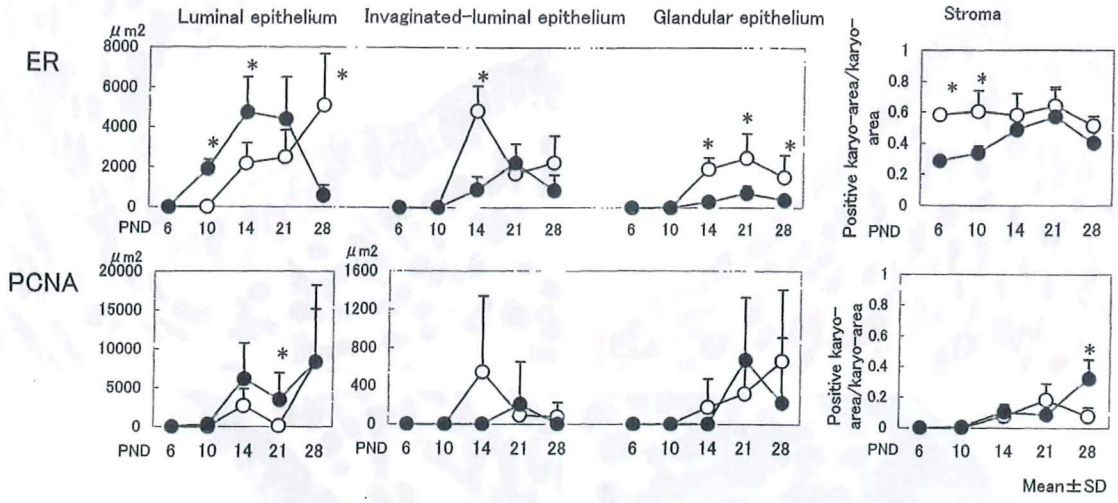

FIGURE 4.—Time course alteration in areas of nuclei positive for ER and PCNA in the luminal, invaginated-luminal, and glandular epithelium and stromal cells. Open and closed circles are for the control and OP-treated groups, respectively. $*p < 0.05$.

appear at various days from PND7 to PND15 (6, 14), but was evident at PND14 in our study, suggesting a dependence on the strain difference as reported in mice (3), or the sensitivity of the detection method between immunohistochemical and radioimmunoassay techniques (14).

In our normal Donryu rats, ER expression in the glandular epithelium began at PND14, being coincident with the time for differentiation of uterine glands, when the luminal epithelium invaginates into the stroma. After PND14, ER expression and PCNA positive cells were concurrently increased in the invaginated-luminal and glandular epithelium. In the OP-treated group, however, gland formation was delayed and inhibited as reported previously by us (22) and other investigators (6–8, 26), although ER expression and PCNA positive cells could be observed in mature glandular epithelium. The decrease of positive areas for ER and PCNA in the glandular epithelium of the OP-treated group can be considered to have resulted from depression of gland-genesis.

In contrast, ER expression in stromal cells of the control group was observed at PND6, similar to many other reports (13, 14, 16, 17, 37), although no induction of PCNA positive cells was detected. OP treatment suppressed ER expression without PCNA alteration, in line with data for mice neonatally exposed to DES (40). The role of ER expression in uterine stromal cells during the perinatal period is not fully understood, although it has been considered to be crucial for proliferation of luminal epithelium in response to epithelial-stromal interaction.

FIGURE 5.—Apoptotic cells in uteri of control (a) and OP-treated rats (b) at PND14. In the OP-treated group, apoptotic cells in the luminal epithelium (arrowheads) are slightly increased as compared with the control group case. ×200.

Our present results indicate that ER expression in both luminal and glandular epithelium is strongly linked with cell proliferation. OP affected the developing uteri at PND10, although routine assessment using H-E staining did not detect any early change at this time point. The effects on ER expression in the uteri are suggested to be a direct estrogenic action of OP, because ovarian-derived endogenous estrogen is very low in this period (13). Fishman et al reported that endogenous estrogens are the primary mediators of uterine growth but not differentiation (14). Our results for inhibition of gland-genesis and abnormal expression of ER and PCNA in the uteri suggest that neonatal exposure to a high dose of OP affects uterine growth and differentiation, in line with other studies (16, 40, 41).

A long-standing paradox is why ER is present at significant levels during the neonatal period, while serum estrogen is very low and the neonatal uterus does not show a full uterotropic response to E2 (13, 14). A previous study using neonatally ovariectomized rats demonstrated that ovary-derived endogenous estrogen is not necessary for uterine differentiation (33). Quite recently, studies using ER knockout mice (ERKO mice) have brought exciting progress in research on ER (12, 24, 25), indicating that the presence of ER in stromal cells is essential for proliferation of epithelial cells induced by E2 treatment (12). Greco et al (16) demonstrated immunohistochemically that estrogen (DES) might affect ER-negative uterine epithelial cells via adjacent ER-positive connective tissue cells, resulting in induction of ER expression in the epithelium of the developing mouse uterus. The suppression of ER expression evident in stromal cells up to PND 10 in our present study might be evidence that OP-treatment affects differentiation of the developing uteri in this way. Recently, other estrogen-mediated factors in the uteri have been investigated (24, 41, 42). Neonatal exposure to DES induced c-fos and p21 expression and decreased apoptosis in the luminal epithelium of mice, indicating suppression of cell cycling of endometrial epithelial cells and induction of abnormal uterine differentiation (41). Our study did not reveal any alteration in the proto-oncogene c-fos or mitotic inhibitor p21, although the apoptotic index in the luminal epithelium appeared slightly elevated in the early treatment period. Other studies also demonstrated estrogen-independent activation by insulin-like growth factors 1 (IGF) or epidermal growth factors (EGF) (24). Our results showing no relationship with EGF expression might provide support for the finding in ERKO mice that EGF is not an important factor for ER expression and subsequent cell proliferating activity mediated by E2 (24).

Effects of perinatal exposure to estrogens including EDCs on uterine carcinogenesis constitute an important social issue. Neonatal exposure to tamoxifen or DES is well known to result in development of uterine or vaginal cancer in human beings or rodents (1, 19, 23, 28–30, 34). Our previous study indicated that uteri of rats exposed to high-dose OP showed intraluminal epithelial hyperplasias at 8 weeks of age (22), similar to preneoplastic lesions for uterine adenocarcinomas in aging Donryu rats (27). Regarding mechanisms underlying such effects, one of the most crucial pathways is alteration of the developmental pattern of hormonal secretion, presumably due to a hypothalamo-pituitary-ovarian disorder, resulting in polycystic ovary with an increased E2:progesterone (E:P) ratio. A high occurrence of spontaneous endometrial adenocarcinomas in the Donryu strain, associated with a hormonal disturbance, high E:P ratio has been reported (27). As another pathway, abnormal differentiation of uteri with abnormal ER expression might modulate directly uterine response to E2. These two pathways suggest that perinatally exposure to OP might lead to development of uterine tumors in Donryu rats through both abnormal uterine differentiation with alteration of ER expression and an altered hormonal status. Therefore, we hypothesize that abnormal development and differentiation of the rat uteri caused by neonatal exposure to OP might modify the occurrence of uterine adenocarcinomas. To clarify this point, additional studies on uterine carcinogenesis in Donryu rats treated with OP neonatally are now underway.

In conclusion, the present study demonstrates that neonatal exposure to OP causes early and enhanced ER expression followed by increased cell proliferating activity in the luminal epithelium, along with suppression of ER and cell proliferation in the glandular epithelium with inhibited gland-genesis. OP treatment also suppressed ER expression in stromal cells at an early age. These results suggest that neonatal exposure to high dose of OP might induce abnormal differentiation of the rat uteri via abnormal ER expression and subsequent change of cell proliferation activity in the endometrium.

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Case Report

Sebaceous Gland Metaplasia in a Mammary Fibroadenoma Developing in a Female Donryu Rat

Takasumi Shimomoto[1,2], Midori Yoshida[1], Masakazu Takahashi[1], and Akihiko Maekawa[1]

[1]Department of Pathology, Sasaki Institute, Tokyo 101–0062, Japan
[2]Department of Toxicology, Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd., Tokushima 771–0192, Japan

Abstract: A mammary fibroadenoma with prominent sebaceous gland-like structures in a female Donryu rat aged 67 weeks was immunohistochemically investigated. The tumor demonstrated a mixture of both epithelial and connective tissue components, the former having both single- and multi-layered patterns. Characteristic sebaceous gland-like structures were apparent in connection with multi-layered epithelium. Area of single-layered epithelium comprised well-differentiated myoepithelial cells positive for alpha-smooth muscle actin (alpha-SMA), similar to mammary ducts. In contrast, many epithelial cells on the basal sides of multi-layers were negative for alpha-SMA, but positive for cytokeratin 14 (CK14), suggesting pluripotency. From these results, we diagnosed this case as a mammary fibroadenoma with sebaceous gland metaplasia. (J Toxicol Pathol 2002; **15**: 73–77)

Key words: fibroadenoma, mammary gland, sebaceous gland metaplasia, spontaneous, Donryu rat

Mammary fibroadenoma is one of the most common spontaneous tumors in aged Sprague-Dawley, Fischer-344, and Donryu rats[1–3]. It is characterized by proliferation of both epithelial and fibrous connective tissue components with no atypia. As a variant, rat fibroadenomas with squamous epithelium or sebaceous cell-like structures have been previously described[4], although they are very rare. Recently, we encountered a mammary fibroadenoma with prominent sebaceous gland-like elements in a female Donryu rat. In this report, we described its morphological characteristics, including histochemical and immunohistochemical features, and discussed the origin of sebaceous gland-like structures.

The animal was one of 39 female rats given a single dose of 200 mg *N*-propyl-*N*-nitrosourea (PNU) per kg body weight by stomach tube at 10 weeks of age. The animal was euthanized at 67 weeks of age on becoming moribund. At necropsy, a large subcutaneous mass was found, in addition to an abscess of the lung, hematoma of the uterus, and enlargement of the spleen. Animal care and use followed the NIH Guide for the Care and Use of Laboratory Animals.

All lesions and major organs were fixed in 10% neutral buffered formaldehyde solution and processed routinely for histopathological examination after H-E staining. In addition, deparaffined sections of the subcutaneous mass were processed for Masson's trichrome histochemistry, and for immunohistochemical analysis using mouse monoclonal antibodies against alpha-smooth muscle actin (alpha-SMA; clone 1A4, DAKO Japan, Kyoto) and cytokeratin 14 (CK14; clone LL002, Novocastra Laboratories Ltd, Newcastle upon Tyne). For this latter, sections were incubated with one of the antibodies overnight at 4°C, and subsequently with secondary antibodies conjugated to peroxidase labeled-dextran polymers (EnVision+, DAKO Japan). Normal sebaceous glands in a female Donryu rat aged 12 weeks were examined immunohistochemically in the same manner for comparison with the present case.

Histologically, the subcutaneous well-circumscribed tumor tissue compressing the adjacent normal tissue was composed of proliferation of both epithelial cells resembling mammary ducts and abundant connective tissue surrounding the epithelial components (Fig. 1a). In the epithelial component, both single- and multi-layered patterns were observed (Fig. 1a), with transitions between the two in part. In the single-layered epithelium, fusiform or stellate myoepithelial cells predominated but they could not been detected in multi-layered areas. There were no cellular atypism and few mitotic figures of epithelial cells.

In the present case, the most striking finding was the presence of sebaceous gland-like structures in association and connecting with multi-layered epithelium (Fig. 1b).

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Mailing address: Takasumi Shimomoto, Department of Pathology, Sasaki Institute, 2–2 Kanda-Surugadai, Chiyoda-ku, Tokyo 101–0062, Japan
TEL: 81-3-3294-3286 FAX: 81-3-3294-3290
E-mail: pathol@sasaki.or.jp

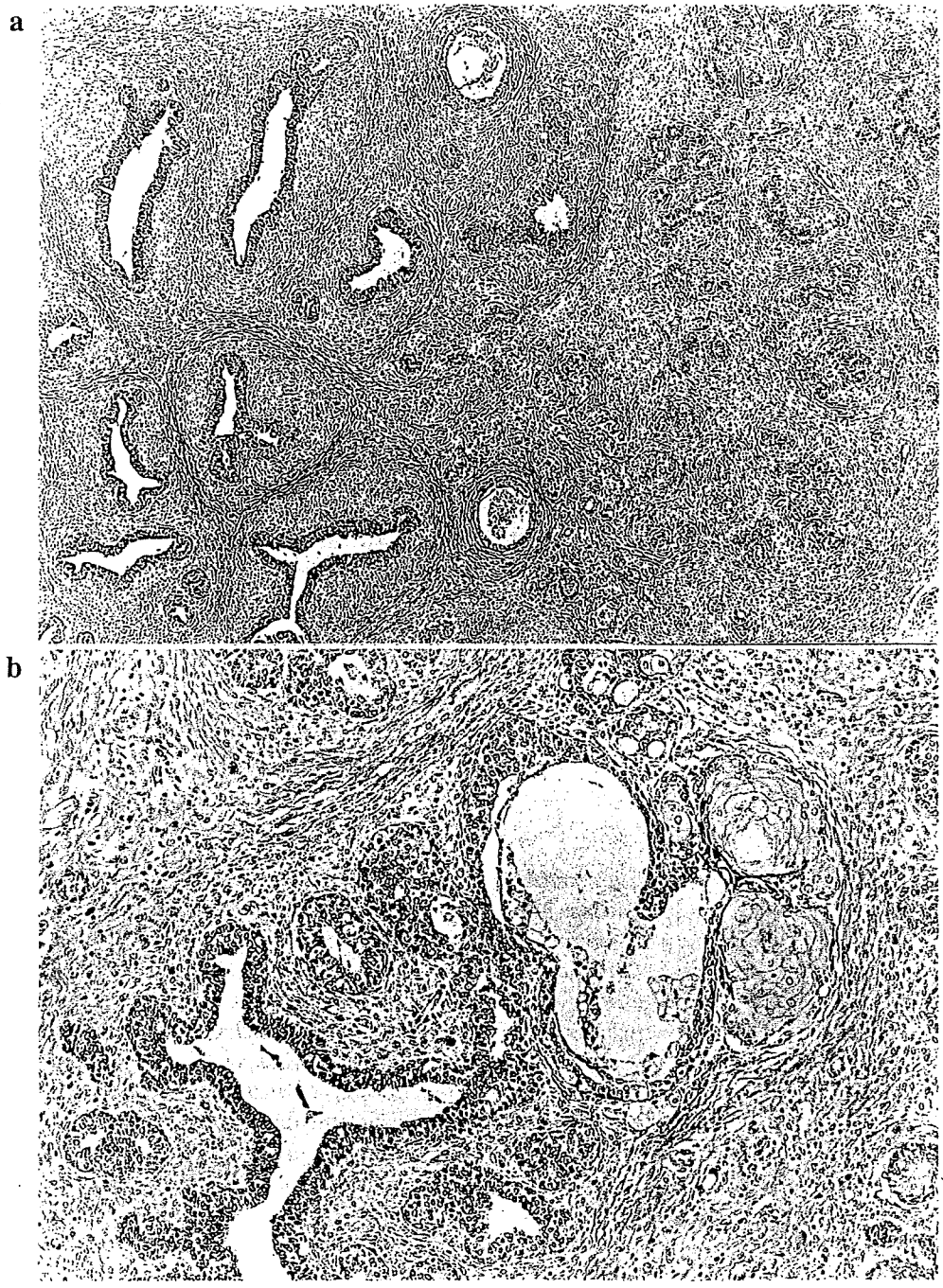

Fig. 1. a) The subcutaneous mass is composed of both epithelial and connective tissue components, the former presenting as ductal structures resembling mammary ducts, with large amounts of surrounding connective tissue. Single-layered (right) and multi-layered (left) epithelia are observed. × 50. H-E. b) Sebaceous gland-like structure observed in association with and connected to a multi-layered area. × 125. H-E.

Many of the component cells possessed finely granular to foamy cytoplasm, with basaloid cells having hyperchromatic nuclei and scant cytoplasm at the periphery, resembling their counterparts in normal sebaceous glands.

Histochemical analysis by Masson's trichrome revealed both epithelial components to be surrounded by large amounts of collagen fibers. The immunohistochemical results are summarized in Table 1. In our case, well-differentiated myoepithelial cells along the single-layered epithelium were generally positive for alpha-SMA (Fig. 2a), but only a few positive cells were detected in the multi-layered areas (Fig. 2b). Cells in the sebaceous gland-like structures as well as normal sebaceous glands in a female Donryu rat were negative for alpha-SMA.

Table 1. Summary of Immunohistochemical Characteristics —Alpha-SMA and CK14—

Antibody	Normal human and rat mammary glands*		Pluripotent cells in human mammary glands**	The present case						Normal rat sebaceous glands
				Single-layered epithelium		Multi-layered epithelium				
	Myo.[1]	Basal[2]		Myo.	Luminal[3]	Myo.	Epithelial cells in the basal side	Epithelial cells in the luminal side	Sebaceous gland-like structure	
Alpha -SMA	Y	No data	N	Y (+)	N	Y (±)	N	N	N	N
CK14	Y	Y	Y	Y (±)	Y (±)	Y (±)	Y (++)	Y (±)	Y (++)	Y

*: According to the literatures[5-12]. **: According to the literature[7]. No data: No record in the literature to our knowledge. [1]: Myoepithelial cells. [2]: Basal epithelial cells. [3]: Luminal epithelial cells. Y: Specific response to antibodies. ±: A few cells responsive, or almost all cells weakly responsive. +: Almost all cells responsive. ++: Almost all cells responsive in wide area. N: No response to antibodies.

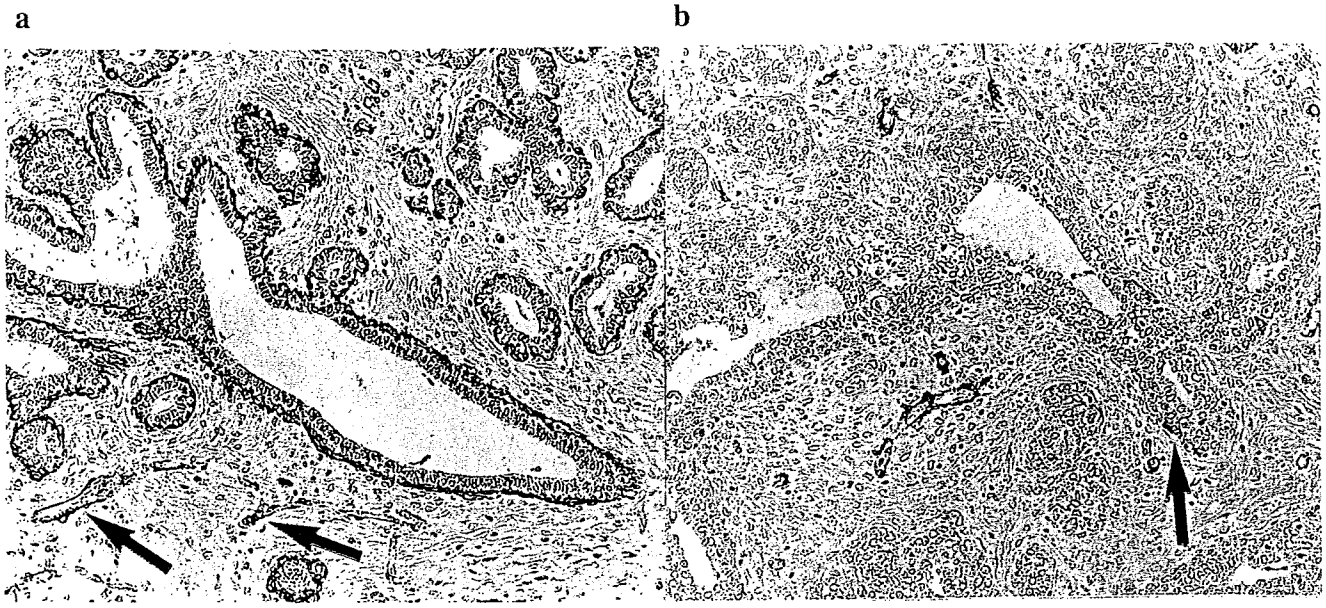

Fig. 2. a) Myoepithelial cells lining the single-layered epithelium are positive for alpha-SMA. Smooth muscle fibers in the blood vessel are also positive (arrows). × 152. Alpha-SMA. b) A few myoepithelial cells along multi-layered epithelium are positive for alpha-SMA (arrow). × 152. Alpha-SMA.

With CK14, myoepithelial cells in the single-layered epithelium were weakly positive (Fig. 3a). While elements on the basal sides of multi-layered epithelium were strongly positive (Fig. 3b), positive cells were found scattered in the luminal epithelium of both epithelial types (Figs. 3a, b). Cells of the sebaceous gland-like structure as well as the normal sebaceous gland were strongly positive for CK14 (Figs. 4a, b), along with the Malpighian stratum including basal cells in the normal skin (Fig. 4b).

The histopathological results described above demonstrated that the present case should be diagnosed basically as a mammary fibroadenoma. While there were obvious differences from the typical tumor, we could rule out the possibility of a mixed mammary fibroadenoma and sebaceous gland adenoma, because of the clear connections between multi-layered epithelium and the sebaceous gland-like structures.

The present fibroadenoma case was atypical in having both single-layered and multi-layered patterns, the former predominating in most cases in rats. Immunohistochemically, in human and rat mammary glands, myoepithelial cells are positive for alpha-SMA[5-7], and basal epithelial/myoepithelial cells are reported to be positive for CK14[7-12]. In the single-layered epithelium of the present case, myoepithelial cells were similarly positive for alpha-SMA, in line with expectation. Interestingly, however, many epithelial cells in the basal side of the multi-layered epithelium were positive for CK14 but negative for alpha-SMA. The basal layer in rat

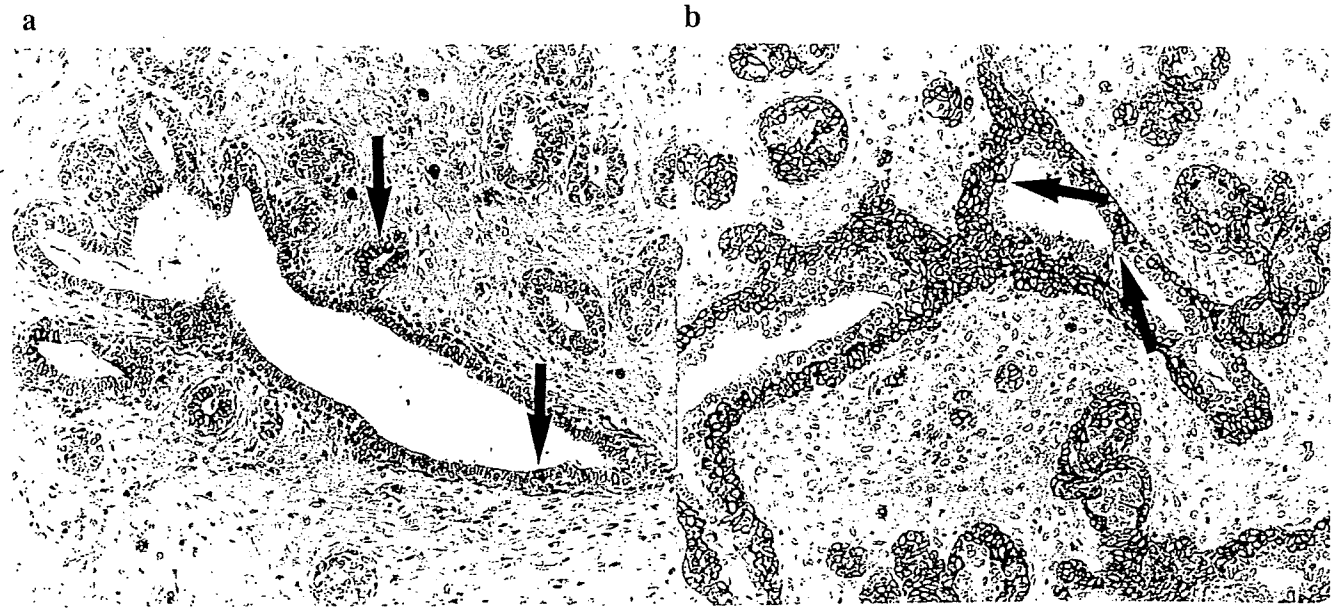

Fig. 3. a) Myoepithelial cells lining single-layered epithelium are weakly positive for CK14. Some of the luminal cells are also positive (arrows). × 152. CK14. b) On the basal sides of the multi-layered epithelium, large numbers of epithelial cells are positive for CK14. Some of the luminal cells are also positive (arrows). × 152. CK14.

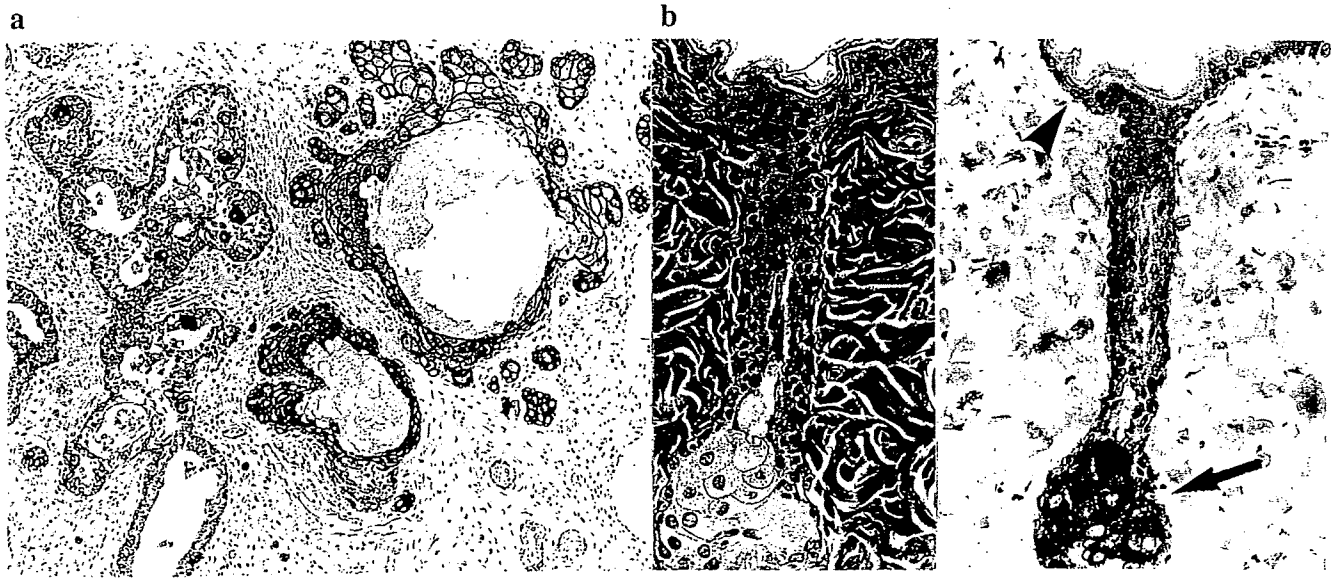

Fig. 4. a) Sebaceous gland-like structures partially positive for CK14. × 95. CK14. b) Normal sebaceous gland in a female Donryu rat aged 12 weeks. The sebaceous gland is positive for CK14 (arrow) as well as cells of the Malpighian stratum including basal cells in the skin (arrowhead). × 251. Left: H-E. Right: CK14.

mammary ducts is considered to contain pluripotent cells capable of developing into either luminal epithelial or myoepithelial cells[13]. In epitheliosis of human mammary glands, some intraluminal proliferative cells are positive for CK14 and negative for alpha-SMA, and it has been suggested that these are post-stem or intermediate cells which generate the luminal epithelium[7]. Our results thus indicate that the CK14-positive and alpha-SMA-negative cells on the basal side of multi-layered epithelium might be similarly pluripotent and some of them giving rise to sebaceous gland-like structures. The lack of malignant features, connection with the multi-layered epithelium and similarity to normal sebaceous glands support the conclusion of metaplasia.

In conclusion, we diagnosed this case as a mammary fibroadenoma with sebaceous gland metaplasia. We could not find similar mammary fibroadenomas in other rats of the

same group, although a total of 14 lesions were observed in the 39 rats of the group, suggesting that the sebaceous gland-like metaplasia was spontaneous in nature.

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Uterine Adenocarcinoma in *N*-Ethyl-*N'*-nitro-*N*-nitrosoguanidine-treated Rats with High-dose Exposure to *p-tert*-Octylphenol during Adulthood

Shin-ichi Katsuda,[1] Midori Yoshida,[1] Hiroyuki Kuroda,[1] Jin Ando,[1] Masakazu Takahashi,[1] Yuji Kurokawa,[1] Gen Watanabe,[2] Kazuyoshi Taya[2] and Akihiko Maekawa[1,3]

[1]*Department of Pathology, Sasaki Institute, 2-2 Kanda Surugadai, Chiyoda-ku, Tokyo 101-0062 and* [2]*Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509*

Since many risk factors are associated with the development of uterine adenocarcinomas in humans, the etiology is unclear in most cases, although it has been pointed out that estrogen may play essential roles. To clarify the effects of exposure to *p-tert*-octylphenol (OP), an environmental xenoestrogen, on uterine carcinogenesis, adult Donryu rats were initiated with a single intrauterine treatment of *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) at 11 weeks of age and exposed thereafter to 100 mg/kg OP by s.c. injection until 15 months of age. Adult ovariectomized (OVX) rats were also treated in a similar way. OP had no effect on occurrence of persistent estrus in middle age, although uterotrophic effects were obvious in OVX rats. At the termination, development of uterine adenocarcinomas was significantly increased in animals exposed to OP during adulthood. No tumors, but a few focal hyperplasias, developed in OVX rats. These findings suggest that OP has tumor-promoting effects on ENNG-treated endometrium of rats, possibly due to direct action on the uterus, as indicated by the uterotrophic effect when a high dose of OP was given. The results provide clues to the mechanisms of influence of hormonal disrupters on uterine carcinogenesis.

Key words: Octylphenol — Rat — Uterine adenocarcinoma — Adulthood

Recently, the possible adverse consequences arising from the release of man-made substances with estrogenic, anti-estrogenic or androgenic properties, so-called endocrine disrupting chemicals (EDCs), into the environment have become an important social issue. *p-tert*-Octylphenol [OP; *p*-(1,1,3,3-tetramethylbutyl)phenol, Fig. 1], one of the alkylphenols (APs), is listed as an EDC with estrogenic activity *in vitro*[1,2] and *in vivo*.[3,4] Environmental OP is thought to be derived from biodegradation of non-ionic surfactants, alkylphenol polyethoxylates (APEOs),[5] and is found in the sludge of sewage-treatment plants as well as in the river and sea sediments.[1,6] It has been pointed out that human exposure to OP may occur not only through drinking water extracted from polluted rivers and foods from fields contaminated with sewage sludge, but also by contact with manufactured and/or breakdown products, such as absorption through skin from shampoos and cosmetics, or inhalation and ingestion from pesticide sprays.[7] While APs, including OP, exist at only very low concentrations in the environment (below 1 μg/liter in water in Europe)[8] and are markedly less estrogenic than estradiol-17β (E2), this does not rule out potential toxicity of chronic exposure to animals and human beings, taking into account the evidence of bioaccumulation in fish.[9]

Carcinogenicity is the most important possible adverse consequence of chemicals including EDCs. In fact, it has been hypothesized that environmental estrogens, including APs, may be causative agents for breast cancer in humans.[10] The uterine adenocarcinoma is one of the most common malignant tumors in women.[11] While its etiology remains largely unclear, it has been pointed out that hormones such as estrogen may play essential roles.[12–15] Menoxenia, polycystic ovary syndrome, chronic anovulation, estrogen replacement therapy, obesity, hypertension, diabetes, and the nulliparous state have been listed as risk factors.[16–20] Recently, epidemiological evidence has accumulated with regard to endometrial cancers as second primaries after the use of tamoxifen, an anti-estrogen, for the treatment of breast cancer.[21] In experimental studies, however, there is only limited evidence that environmental chemicals/hormones induce uterine adenocarcinomas in rodents, as reviewed recently.[22] We have documented that the Donryu rat is a high incidence strain for spontaneous development of uterine adenocarcinomas, associated with a hormonal imbalance characterized by an increased estrogen-progesterone ratio.[23–25] The incidence of spontaneous uterine adenocarcinomas in this rat strain showed a tendency to decrease in animals having reproductive experience, compared to the nulliparous case, suppression being associated with changes in the hormonal milieu.[26] These results indicate that the Donryu rat may be a good animal model for uterine adenocarcinoma linked to endogenous estrogens in humans. An elevated incidence of such tumors develops in this rat strain with a single intra-uterine administration of *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine

[3] To whom requests for reprints should be addressed.
E-mail: amaekawa@sasaki.or.jp

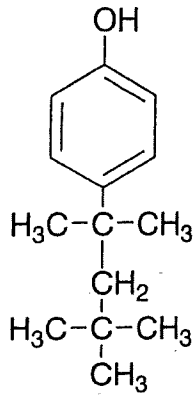

Fig. 1. Chemical structure of *p-tert*-octylphenol.

(ENNG) and a two-stage rat uterine carcinogenesis model has already been established.[27] This animal system has advantages for clarification of the tumor-promotive effects of long-term exposure to estrogens and/or estrogenic compounds during adulthood, with acceleration of cell proliferative activity in the uterus and/or indirect effects such as perturbation of endocrine regulation.[22]

We performed a series of comprehensive experiments to study the influence of OP on uterine carcinogenesis using Donryu rats. In the rats, the first ovulation, termed puberty, usually begins at 5 or 6 weeks of age. Thereafter, a 4- or 5-day sexual cycle is repeated and the rat after 11 or 12 weeks of age has fully grown to be an adult. In the current study, to clarify the effects of long-term OP exposure during adulthood on uterine carcinogenesis, both ovary-intact and ovariectomized (OVX) adult female animals, initiated with a single intra-uterine administration of ENNG at 11 weeks of age, were given a high dose of OP s.c. until 15 months of age. In the adult OVX rat, 2 consecutive injections of 100 mg/kg OP caused marked estrogenic effects.[4] We selected this dose to confirm the maximum effect of OP on the uterine carcinogenesis. As suggested by Certa *et al.*,[8] to minimize the metabolism of OP during first passage through the liver, animals were treated with OP by s.c. administration.

MATERIALS AND METHODS

Animals and housing conditions Female Crj:Donryu rats were obtained from Charles River Japan, Inc. (Kanagawa). They were housed in plastic cages and kept in an air-conditioned animal room under constant conditions of 24±2°C and 55±10% humidity with a 12 h light/dark cycle, and maintained on basal diet, CRF-1 (Oriental Yeast, Inc., Tokyo) and tap water *ad libitum*. Animal care and use followed the NIH Guide for the Care and Use of Laboratory Animals.

Experimental design Four experimental groups were prepared, as shown in Fig. 2. Groups 1 and 2 were ovary-intact, and groups 3 and 4 were OVX adult rats with or without long-term OP treatment from 11 weeks of age until 15 months of age.

Groups 1 and 2: ovary-intact rats with or without OP treatment When rats were 11 weeks of age, at which time they are more sensitive to chemical carcinogens than when older,[22] a single dose of 20 mg/kg ENNG (Nacalai Tesque, Inc., Kyoto) dissolved in polyethylene glycol was given into a unilateral uterine cavity using a stainless catheter via the vagina, as reported previously.[27] Subsequently, rats were given s.c. injections with dimethylsulfoxide (DMSO) (group 1) or OP (Wako Pure Chemical Ind., Ltd., Osaka). The dose of OP was 100 mg/kg body weight, and this was applied 5 times/week for the first 2 weeks, 3 times/week for the next 11 weeks, and 2 times/week thereafter. In our previous study, the dose was ascertained to exert strong uterotrophic effects on OVX rats with treatment of OP for 2 weeks,[4] and after that, dosing times of the treatment were reduced because irritative responses such as induration and erythema were evident at the injection sites. For sequential histological observations and hormone assays, 6–8 animals in each group were sacrificed at 9 and 12 months of age (6 and 9 months after the beginning of dosing, respectively). All surviving rats (23–26 animals in each groups) were killed at 15 months of age (12 experimental months).

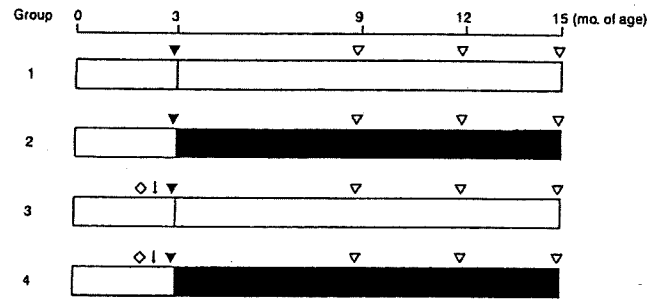

Fig. 2. Experimental design for examination of the effects of *p-tert*-octylphenol (OP) on uterine carcinogenesis. Intact (groups 1 and 2) and ovariectomized (groups 3 and 4) Donryu rats were initiated with *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) and then received s.c. injections of 100 mg/kg OP or vehicle alone (dimethylsulfoxide) until the end of the experiment (15 months of age). Dosings were made 5 times/week for the first 2 weeks, 3 times/week for the next 11 weeks, and 2 times/week thereafter. ▼ a single intra-uterine application of 20 mg/kg ENNG via the vagina, ▽ sacrifice, ◇ ovariectomy, ↓ priming with s.c. injections of 15 ng of estradiol-17β three times at 13, 14, and 15 days after ovariectomy, ■ s.c. injection of 100 mg/kg *p-tert*-octylphenol (OP). 1, control—ovary-intact animals; 2, OP-exposed—ovary-intact animals; 3, control—ovariectomized animals; 4, OP-exposed—ovariectomized animals.

Groups 3 and 4: OVX rats with or without OP treatment To assess the role of the ovary in uterine carcinogenesis, adult OVX rats initiated with chemical carcinogen were chronically exposed to OP. At 8 weeks of age, ovariectomy was performed by the dorsal route under general ether anesthesia. The success of the operation was confirmed by demonstration of castration vaginal smears characterized by predominant leukocytes with few epithelial cells over at least 4 days. The rats were then primed with s.c. injections of 15 ng of E2 (Wako Pure Chemical Ind., Ltd., Osaka) 3 times at 13, 14, and 15 days after ovariectomy, to increase the sensitivity to estrogens.[28] After 3 weeks (11 weeks of age), 20 mg/kg ENNG was administered into a uterine horn under laparotomy, and thereafter animals were s.c. injected with vehicle alone (group 3) or OP (group 4) in the manner described above and sacrificed following the same schedule (3–6 rats in 9 and 12 months of age, and 15–29 rats in 15 months of age).

Histological examination and hormonal assays All animals were checked for general condition every day and body weights were measured every 2 weeks. Vaginal smears were checked at 4 and 6 months of age and every 3 months thereafter to confirm the estrous cycle stage in groups 1 and 2, and estrous conversion in groups 3 and 4. At necropsy, animals were weighed and then sacrificed by decapitation. Serum samples were collected at necropsy and frozen at −70°C until analysis. The reproductive tract tissues and other representative organs such as the pituitary, lungs, liver, kidneys, and adrenals were quickly removed, weighed and fixed in 10% neutral buffered formalin, and then routinely processed for histopathological examination. Animals found dead or sacrificed when moribund were also autopsied and sampled for histopathology. Each uterus was dissected into about 12 slices in cross-section and proliferative endometrial lesions were classified into three degrees of hyperplasias (slight, +; moderate, ++; severe, +++) and adenocarcinoma using our categories for rat uterine proliferative lesions reported previously.[25] In addition, adenocarcinomas were subdivided into well–moderately (G1–2) and poorly differentiated (G3) types, and also classified as to the degree of invasion; I–II, tumors limited in the uterus; III–IV, tumors invaded into the serosa and/or surrounding adnexae, including cases with distant metastases, in accordance to the simplified FIGO histopathological grades of human uterine cancers.[29] Serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured with NIDDK-rat-FSH and -LH radioimmunoassay (RIA) kits (NIAMDD, NIH, Bethesda, MD).[30] Concentrations of prolactin (PRL), E2 and progesterone were also measured by RIA.[31] Immunoreactive inhibin in the serum was analyzed by double-antibody RIA using a rabbit anti-serum, TNDH-1.[32]

Statistical analysis Values for incidences were statistically analyzed using the Fisher's exact probability test. Other data were analyzed using ANOVA, and post hoc comparisons between OP-treated and control groups were made with Student's t test. A P value less than 0.05 was considered to be statistically significant.

RESULTS

Effects of OP treatment on vaginal smears and uterine weights of ovary-intact and OVX rats At 4 months of age (about 1 month after the beginning of the dosing), the estrous cycle stage could be easily identified from vaginal smears, a precise 4-day cycle being evident in group 1, as shown in Table I. In OP-treated animals (group 2), vaginal cytology of metestrous and/or diestrous stages was significantly disturbed with large amounts of contaminating epithelial cells, although the duration of the estrous cycle was not affected. Table II summarizes data for sequential changes in the incidence of persistent estrus in control (group 1) and OP-treated animals (group 2). Persistent estrus, characterized by vaginal smears exhibiting nucleated epithelial cells and/or cornified cells, began to appear after 6 months of age in both groups, with no difference in incidence between groups 1 and 2 throughout. On the other hand, in the OVX rats, OP injections caused marked uterotrophic effects (group 4; Fig. 3), although relative uterine weights were still only 1/5th to 1/6th of those of

Table I. Effects of *p-tert*-Octylphenol (OP) on Estrous Cyclicity at 4 Months of Age (before the Beginning of Persistent Estrus)

Group	Abnormal estrous cycle/stage	
	Abnormal duration of estrous cycle[a)]	Disturbed estrous cycle stage[b)]
1: Control	0/35 (0)	0/35 (0)
2: OP-treated	3/35 (9)	19/35 (54)**

** Significantly different from group 1 ($P<0.01$).
Numbers in parentheses are percentages.
a) Term shortened or prolonged with estrous cycle less or more than 4 days.
b) Large amounts of contaminating epithelial cells in vaginal smears at metestrous and/or diestrous stages.

Table II. Sequential Changes in Incidences of Persistent Estrus in Ovary-intact Animals

Group	6	9	12	15 (months of age)
1: Control	11/35 (31)	29/35 (83)	27/28 (96)	21/21 (100)
2: OP-treated	16/33 (48)	27/31 (87)	26/28 (93)	26/26 (100)

Numbers in parentheses are percentages.
Vaginal cytology was examined.
OP, *p-tert*-octylphenol.

Table III. Incidences of Uterine Proliferative Lesions

Age	Group	n	–	Hyperplasia				Adenocarcinoma
				+	++	+++	Total	
9 months	1: Control	6	1	5	0	0	5	0
	2: OP-treated	6	2	4	0	0	4	0
	3: Control (OVX)	3	3	0	0	0	0	0
	4: OP-treated (OVX)	6	6	0	0	0	0	0
12 months	1: Control	6	1	2	3	0	5	0
	2: OP-treated	8	0	4	1	0	5	3
	3: Control (OVX)	3	3	0	0	0	0	0
	4: OP-treated (OVX)	7	6	1	0	0	0	0
15 months	1: Control	23	2	2	8	7	17	4
	2: OP-treated	26	0	1	8	5	14	12[a)]
	3: Control (OVX)	15	15	0	0	0	0	0
	4: OP-treated (OVX)	29	25	3	1	0	0	0

a) Incidence of adenocarcinomas is significantly different from the controls ($P<0.05$).
OVX, ovariectomy; OP, *p-tert*-octylphenol.

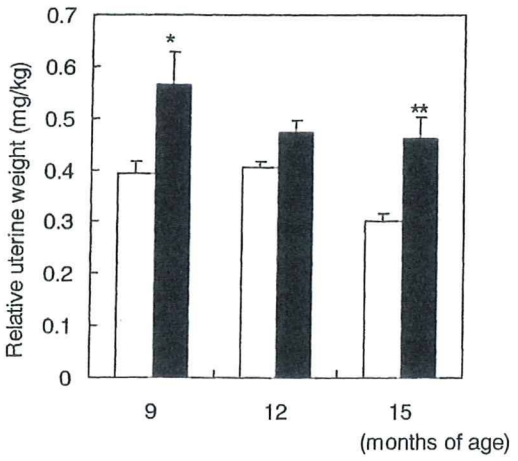

Fig. 3. Uterotrophic effects in adult ovariectomized rats treated with *p-tert*-octylphenol (■ group 4), and the controls (□ group 3). Data are expressed as mean±SE values. * $P<0.05$, ** $P<0.01$.

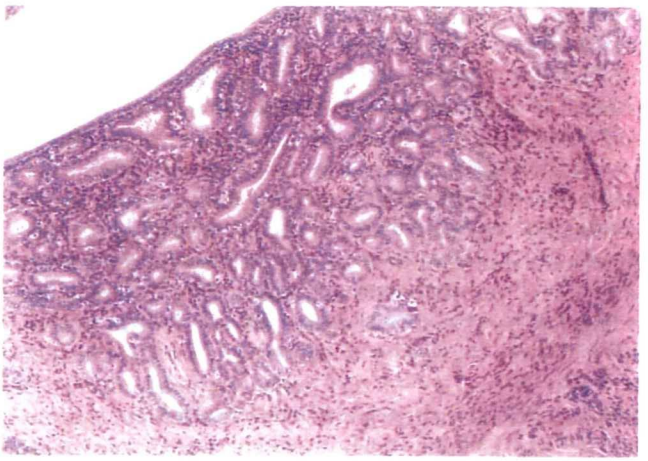

Fig. 4. Endometrial hyperplasia (moderate, ++) with focal proliferation of uterine glands found in a rat of group 2. ×90.

ovary-intact groups with or without OP (groups 1 and 2) (data not shown). Estrus conversion in vaginal smears appeared in 83% of OP-treated animals (group 4) at 4 months of age, but thereafter almost all animals showed no estrus.

Uterine proliferative lesions and other histopathological findings A comparison of sequential development of uterine proliferative lesions in control and OP-exposed rats (groups 1 and 2) is given in Table III. In both controls and rats treated with OP (groups 1 and 2), endometrial hyperplasias increased in number and severity with age (Table III), most being focal proliferations of uterine glands with apparent duct structures in the stroma of the endometrium (Fig. 4).

Uterine adenocarcinomas were significantly increased in ovary-intact rats treated with OP (group 2), as compared with group 1 ($P<0.05$) (see Table III). The results of subclassification of adenocarcinomas at 15 months of age, regarding degree of differentiation and invasion, are shown in Table IV. All adenocarcinomas in group 1 and almost adenocarcinomas in group 2 were of well-differentiated type (Fig. 5), but 3 cases in group 2 were of poorly

Table IV. Degrees of Differentiation and Invasion of Uterine Adenocarcinomas Found at 15 Months of Age

Age	Group	Total	Differentiation[a]		Invasion[b]	
			G1–2	G3	I–II	III–IV
15 months	1: Control	4	4 (100)	0 (0)	4 (100)	0 (0)
	2: OP-treated	12	9 (75)	3 (25)	10 (83)	2 (17)

Numbers in parentheses are percentages.
Adenocarcinomas were classified in accordance with the simplified FIGO histopathologic grades of human uterine cancers.
a) Histopathological grades of uterine adenocarcinomas by tumor differentiation. G1–2, well to moderately differentiated; G3, poorly differentiated.
b) Degree of invasion of uterine adenocarcinomas. I–II, tumors limited to the uterus; III–IV, tumors invading into the serosa and/or surrounding adnexae, including intra-abdominal and distant metastases.
OP, *p-tert*-octylphenol.

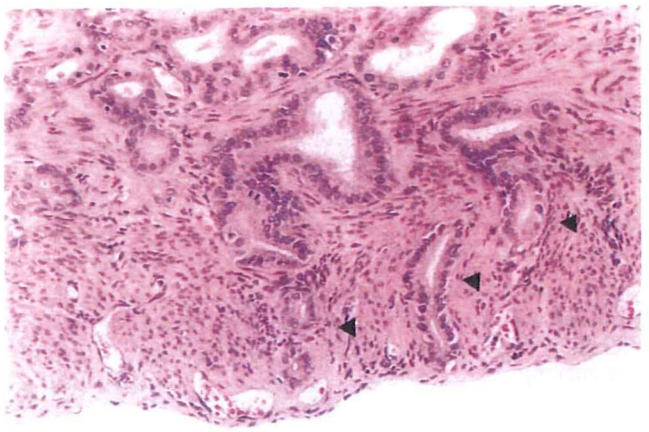

Fig. 5. Well-differentiated uterine adenocarcinoma found in a rat of group 2. Arrowheads show tumor cells invading the myometrium. ×180.

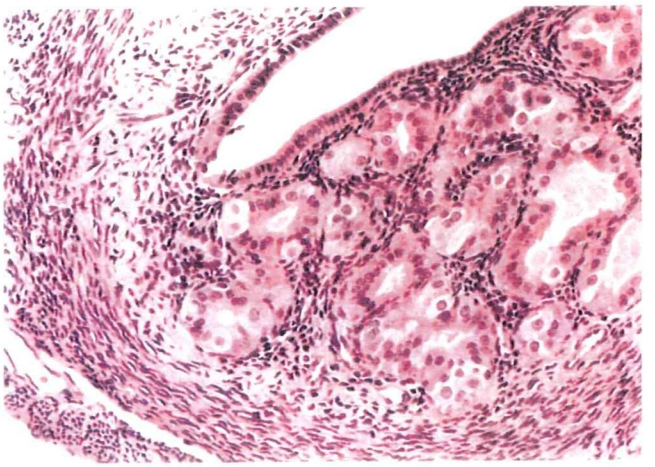

Fig. 6. Endometrial hyperplasia (weak, +) with focal proliferation of uterine glands found in a rat of group 4. ×210.

differentiated type, invading into the serosa of the corpus uteri and/or adnexae. No tumors were observed in OVX rats with or without OP treatment (groups 3 and 4) at any age examined. Although all uteri were thin and atrophic in these animals, five OP-exposed uteri (group 4) at 15 months of age had focal hyperplasias, ranging from slight to moderate (Fig. 6).

At terminal necropsy, ovarian atrophy/cyst formation and lack of corpora lutea were observed in almost all animals of groups 1 and 2.

Endocrine environment Hormonal profiles of serum FSH, LH, PRL, E2, progesterone, and inhibin in groups 1 and 2 are shown in Fig. 7. Although FSH levels were not different between the two groups, LH levels in OP-treated rats were significantly lower than the control values. As compared to the controls, the serum E2 level in OP-treated rats was lower at 9 months of age. Although serum progesterone levels did not significantly differ between the groups, serum inhibin levels in OP-treated rats (group 2) were higher at 12 months of age and thereafter.

DISCUSSION

Estrogens are well established to be important etiological agents for uterine carcinogenesis in humans.[12–15] Although the exact roles still remain to be detailed, the tumor-promotive effects of up-regulation of cell proliferation have long been suggested. Natural and synthetic estrogenic hormones express their effects mainly by binding to estrogen receptors. OP is a typical putative endocrine disrupter, binding to ER and exerting estrogenic effects.[1, 33] Recently we determined that OP increased the proliferation of endometrial cell components in adult OVX rats, in a dose- and period-dependent manner.[4] Until now,

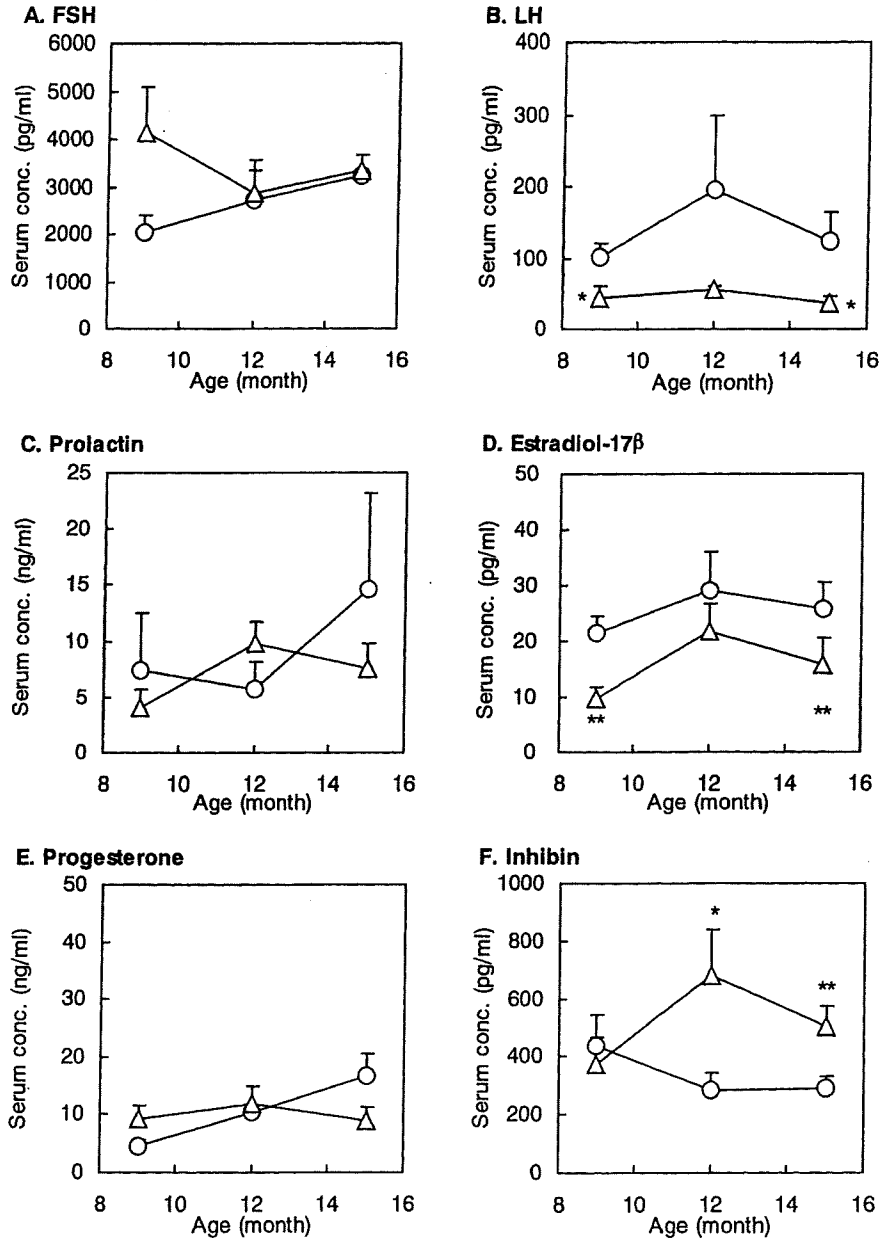

Fig. 7. Serum hormone levels in rats of the control (group 1, ○) and OP-treated (group 2, △). Animals were sacrificed at 9, 12, and 15 months of age and serum samples were collected. Data are expressed as mean±SE values. * $P<0.05$, ** $P<0.01$. OP, *p-tert*-octylphenol.

however, there has been no report concerning carcinogenic effects of OP on the female genital tract, including the uterus, in rodents. Thus, we performed the present study, in which a high dose was administered during adulthood. The present results provide support for the hypothesis that exposure to high doses of OP may result in promotive effects on uterine carcinogenesis in rats.

In considering the carcinogenic effects of hormones and/or hormone-like chemicals such as OP, it is very important to clarify whether the mechanisms involve a direct action on the target organs or an indirect influence via perturbation of hormonal regulation. In the present study, long-term OP exposure caused apparent uterotrophic effects in OVX rats. On the other hand, in ovary-intact rats, age-related persistent estrus was not perturbed by OP treatment at 6 months of age and thereafter, although abnormal estrous cycling was significantly increased at 4 months of age. Age-related ovarian atrophy/cyst formation was evident in ovary-intact groups (groups 1 and 2), but there were no differences in incidence or morphology. These findings suggest that dosing with OP during adulthood in this experiment had clear uterotrophic

effects, but little influence on hypothalamic function. There is some experimental evidence of the development of tumors following treatment with xenoestrogens, irrespective of endogenous estrogen levels, e.g., mammary tumor by estradiol benzoate in the rat,[34] and renal tumor by diethylstilbestrol (DES) in the hamster.[35] Accordingly, OP may impact directly upon the uterus as a xenoestrogen, the tumor-promotive effect depending on its estrogenic activity additional to that of endogenous hormones. The fact that adenocarcinomas were not induced in OVX rats exposed to OP indicates that endogenous ovarian hormones are needed for uterine tumor development. After 9 months of age, the hormonal environment was affected by OP exposure. Low levels of E2 in rats treated with OP during adulthood might be induced by negative feedback due to OP exposure, as LH levels were consistently lower in those animals. In the present study, the relative estrogen level (E2/progesterone ratio), known to be very important for uterine carcinogenesis in humans and rodents,[22] was low at 15 months of age. Therefore, it should be clarified whether an additional hormonal effect of OP might play a key role in uterine carcinogenesis or not, although ovarian hormones are essential to tumor development as mentioned above. The persistent-estrous state in aging rats, characterized by a lack of ovulation and absence of estrous cycles, was associated with enhanced inhibin subunit mRNA expression in large and anovulatory follicles of the ovaries.[36] High levels of inhibin in rats treated with OP during adulthood may indicate that residual granulosa cells in the atrophic ovaries possess the ability to secrete inhibin, and/or that small follicles which lack estrogen-secreting ability might exist.

Previous observations concerning spontaneous uterine adenocarcinoma development in Donryu rats indicated that uterine adenocarcinomas can arise from both lining epithelium and uterine glands, but especially the latter.[25] In the present case of rats treated with OP, endometrial hyperplasias due to focal proliferation of uterine glands were frequently seen. The fact that many tumors were well to moderately differentiated with definite duct-structures closely resembling uterine gland may also have histogenetic significance. On the contrary, neonatal exposure to OP caused marked depression of the number of uterine glands.[37] It was reported that DES-treated mice had decreased numbers of uterine glands, when exposed during neonatal period.[38] Neonatal treatment of rats with tamoxifen induced uterine adenocarcinoma without hyperplasia, and it was concluded that the suppression of uterine-gland genesis by exposure to tamoxifen may account for the low incidence of hyperplasias.[39] Based on this evidence, we hypothesized that OP might affect the uterine gland differently depending upon the age at exposure, and induce different types and incidences of tumors from the present case.

In conclusion, a high-dose OP exposure during adulthood can enhance the development and/or growth of uterine adenocarcinomas in rats. The present evidence shows that a xenoestrogenic compound can act as a tumor promoter through its estrogenic activity, additional to that of endogenous hormones. Since APs including OP exist at only very low concentrations in the environment, the relevance to the human situation of the present findings is not clear. To clarify this point, additional studies are now under way, using lower doses of OP by the oral route.

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