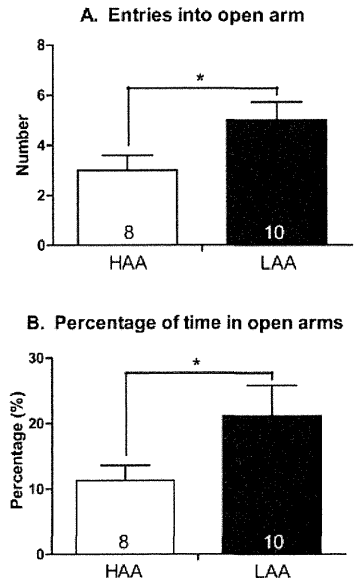

Fig. 3. Behavioral profiles in the elevated plus maze test; (A) frequency of entries into open arm and (B) percentage of time spent exploring the open arms by HAA (white bars, n=8) and LAA (black bars, n=10) males. Numerals in the bars indicate the number of rats in each group. All results are expressed as means ± SEM and asterisks indicate significant difference between the columns ($P<0.05$).

Roman high- (RHA) and low-avoidance (RLA) animal strains, derived from Wistar rats, do not show significant strain differences in the passive avoidance task [15]. These differences among the 3 inbred strains, including the Hatano strain, in the passive avoidance task may have occurred due to different criteria for selection in the shuttle-box task. For the Syracuse rats, 10 pretest trials were performed before the 60 trials of the shuttle-box task [7]. Firstly, they selected only animals that fled to the non-stress chamber fewer than 5 times with a short-latency (5.0 s) during the pretest, and animals showing such a response fewer than 3 times during the last 5 trials of the pretest were selected for the following 60 trial session. Finally, they selected either high or low avoidance animals relative to the generation mean in the 60 trial session as SHA and SLA, respectively. In short, SHA and SLA were selected via the 60 trial session from the population selected by the pretest that did not recognize CS as aversive stress selected [7]. For the Roman rats, high and low avoidance rats were simply selected relative to the generation mean as RHA and RLA, respectively [5]. Thus, freezing animals that had learnt CS might be included in both SLA and RLA. Therefore, the behavioral difference in the passive avoidance task in Syracuse and Roman rats seems to be due to high behavioral inhibition in the low avoidance lines, rather than their low ability in avoidance learning [8–10, 15, 16]. Last but not least, it is strongly indicated that selection and breeding of the Hatano rats were properly carried out on the basis of avoidance learning in the shuttle-box.

For the secondary objective, we examined the emotionality of HAA and LAA rats by open field and elevated plus maze tests. In the present study, the time spent exploring the central area of the open field test was significantly lower in HAA than in LAA rats. Similarly, the number of open-arm entries and the percent time spent in the open arms in the elevated plus maze test were also significantly lower in HAA than in LAA rats. Exploration of the central area in the open field or open arms in the elevated plus maze is regarded as an indication of decreased anxiety-related status of the animal [6, 22, 32]. Therefore, the present results suggested that the male HAA rats are predisposed to high anxiety compared with the male LAA rats. Previous studies demonstrated that Hatano strains have clear strain differences in the functions of the hypothalamo-pituitary-adrenal (HPA) axis [1, 2, 4], which has a pivotal role in physiologic response to stress, learning and anxiety-related status in animals [13, 20, 23, 24, 33, 34, 37, 38]. Corticotropin-releasing hormone (CRH), arginine vasopressin (AVP) and prolactin are known as anxiety-related hormones [18, 23, 25, 38]. For example, exogenous administration of CRH induces anxiety-like behaviors in rats [23]. In Hatano rats, the basal level of CRH content in the paraventricular nuclei (PVN) is significantly lower in HAA than in LAA rats; however, HAA rats showed higher emotional reactivity than LAA rats. Furthermore, the level of CRH content in the PVN showed no significant strain difference after the first session of the shuttle-box task [1]. Additionally, the level of CRH and AVP contents in the amygdala showed no strain difference before and after the first session of the shuttle-box task [1]. Central administration of prolactin engenders anxiolytic-like effects in the elevated plus maze test [38]. In Hatano rats, prolactin secretion as a stress response is signifi-

cantly lower in HAA than in LAA rats [2, 4]. Also, prolactin receptors, especilally long-form receptor expression in the PVN, are not altered in HAA rats but are significantly increased in LAA rats after restraint stress in water [2]. It is known that the long-form receptor is dominant to the short-form receptor, and long-form receptor expression is increased by stress in rats [38]. Also, downregulation of the long-form receptor increases anxiety-like behavior in the elevated plus maze [38]. Thus, we speculated that the strain differences in emotional reactivity in the present results might be due to the differences in endocrine stress response such as prolactin secreting ability and/or sensitivity between HAA and LAA rats, although further evidence is required.

Coping strategy, either active or passive style, is also an important factor associated with emotionality for choosing SSDRs [14, 36], and it would generate diverse behaviors. As mentioned earlier, emotionality affects the results of avoidance behavior; however, there is no coherent relationship with avoidance performance. For example, SLA and RLA rats show higher emotional reactivity, but LAA rats show lower emotional reactivity as compared with their high avoidance counterparts, respectively [7, 17, 36]. This discrepancy is explained by the concept of coping strategy. Passive coping animals with high anxiety may show freezing behavior, and active coping animals with high anxiety show avoidance/escape behavior. Thus, an investigation into the coping style would provide further understanding of the characteristics of Hatano rats.

Further, the total time traveled and the total distance traveled in the open field test were significantly greater in HAA than in LAA rats. These results coincided with the previous report showing that the ambulation length of HAA in the open field test was greater than that of LAA rats at 5 weeks old [29]. In Hatano rats, it is known that HAA rats show higher activity in wheel running than LAA rats, and it was not altered by cross-fostering [28, 29], although the Hatano strain was not selected by the criterion of spontaneous activity [30]. Thus, it is considered that this strain difference is caused by the difference in locomotor activity between HAA and LAA rats.

In summary, the present study clearly demonstrated the high and low avoidance performances of HAA and LAA rats respectively in the different avoidance tasks. The present results suggest that the selection and breeding of Hatano strain rats were properly performed with the criterion of avoidance learning by the shuttle-box task, although other avoidance learning paradigms using non-electric stimuli like predator odor or something else are required to disclose their whole aspects in regard to avoidance performance. Additionally, the HAA strain exhibits higher anxiety-like behaviors compared with rats of the LAA strain. We assume that this strain difference is partly due to the difference in endocrine stress response to a novel environment. Thus, anxiety and its endocrine response, the critical factors for species' survival and conservation, might be associated with acquisition of avoidance learning ability.

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Original Article

Delayed effects of single neonatal subcutaneous exposure of low-dose 17α-ethynylestradiol on reproductive function in female rats

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ABSTRACT -- Delayed effects of exposure to small amounts of estrogenic compounds during the critical period of brain sexual differentiation were investigated by subcutaneous treatment of female Sprague-Dawley rats with 0 (vehicle control), 0.08, 0.4, or 2 μg/kg of 17α-ethynylestradiol (EE) on postnatal day (PND) 1. The treatment did not affect growth and development of the treated animals, and the timings of vaginal opening were similar between the EE-treated and control groups. The animals were periodically examined for the estrous cycle from postnatal week (PNW) 8-9 to PNW 32-33. Patterns of the estrous cycle were similar among the groups until PNW 17. None of the control animals showed persistent estrus until PNW 33. The animals treated with 0.4 μg/kg or more EE showed persistent estrus from PNW 20. The alteration was reflected in the number of days judged as proestrus or estrus, and was found to gradually increase in the EE-treated groups. At necropsy on PNW 32-33, ovulation was not confirmed in most EE-treated animals, even on the day of estrus. In addition, sporadic milk accumulations were observed in the mammary gland of the EE-treated animals. Histological evaluation revealed cystic follicle formation in the EE-treated ovaries and also revealed hyperplasia of mammary glands. Furthermore, ovaries from the animals showing persistent estrus lacked corpus luteum, indicating long-term anovulation. These results clearly show that single exposure to EE during the critical period of brain sexual differentiation can exert effects on reproductive functions at a later period in rats.

Key words: Delayed effect, Ethynylestradiol, Critical period, Brain sexual differentiation, Estrous cycle, Estrogenic compounds

INTRODUCTION

During critical periods of morphogenesis and functional differentiation, animals are substantially sensitive to physiologically active xenobiotics. There are concerns that exposure to such chemicals during these critical periods causes profound adverse effects on exposed animals and humans. In rodents, such as rats and mice, the perinatal period is critical for brain sexual differentiation, and *de novo* synthesized estradiol in the brain by aromatization of testis-derived testosterone is a key signal for masculinization of the male brain. Because estradiol can also masculinize the female brain, exposure of the female brain to aromatizable testosterone or estrogenic compounds, such as synthetic estrogens, xenoestrogens, and phytoestrogens, causes masculinization of female neonates and loses cyclic revolution of estrus after puberty (reviewed in Gore, 2008).

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Although recent studies revealed molecular alterations in the brain after perinatal exposure to estrogenic compounds (Monje *et al*., 2010; Garcia-Galliano *et al*., 2012), studies on the delayed effects of such compounds on development of reproductive function are limited to those by repeated administration during the neonatal period or by transgenerational administration. Briefly, *o,p'*-dichlorodiphenyltrichloroethane (DDT), one of isomers of DDT, induces persistent vaginal estrus in rats when administered subcutaneously during PND 2-4 at dose levels of 0.1 mg/animal or more (Gellert *et al*., 1974). Bisphnol-A (BPA), a monomer used in polycarbonate manufacture, has been extensively studied for its estrogenic properties (reviewed in Vandenberg *et al*., 2009), and has been found to induce persistent vaginal estrus in rats 15 weeks after the day of vaginal opening when administered subcutaneously at a dose level of 50 μg/kg BW during PND 0-3 (Adewale *et al*., 2009). Similarly, BPA alters the estrous cycle of female rats at PND 100 when administered at a dose level of 0.05 or 20 mg/kg BW during PND 1-7 (Monje *et al*., 2010). Methoxychlor is an organochlorine pesticide, and its injection increases irregular estrous cycle when administered to dams from gestational day 19 to PND 7 at a dose level of 100 mg/kg BW (Armenti *et al*., 2008).

In the present study, we therefore evaluated delayed effects of a single low-dose exposure to estrogenic compounds during the critical period of brain sexual differentiation using EE as a model compound. EE is a synthetic estrogen that has been widely used as a constituent of contraceptive formulations. Because EE is orally active, it is used not only for medical purposes but also as a positive control in estrogenic potency studies such as the uterotropic bioassay in the Organization for Economic Co-operation and Development (OECD) test guidelines (Kanno *et al*., 2001, 2003; Owens and Koëter, 2003; Kim *et al*., 2005). The present study involving single administration of EE to neonatal female rats might help to estimate delayed effects of chemical substances showing estrogenic potency at various degrees.

MATERIALS AND METHODS

Chemicals

EE (CAS #57-53-6; purity 99%; Sigma-Aldrich Japan, Tokyo, Japan) was dissolved in ethanol (Wako Pure Chemical Industries, Osaka, Japan) at a concentration of 100 mg/ml, and then the solution was diluted with corn oil (Wako Pure Chemical Industries) to formulate EE to a constant volume of 1 ml/kg.

Animals and housing

Animal experimental procedures were approved by the Committee of Animal Experiment in the Azabu University. Pregnant Sprague-Dawley rats [Crl:CD(SD) IGS] were purchased from Charles-River Japan, (Kanagawa, Japan). They were maintained in an animal facility under controlled conditions (lights on 08:00-20:00; 21 ± 1°C; 50%-60% humidity) in plastic cages with bedding materials (Sunflake; Oriental Kobo, Tokyo, Japan), pellet chow (CE-2; Clea Japan Inc., Tokyo, Japan), and water (tap water; supplied by Sagamihara City, Kanagawa, Japan) *ad libitum*.

Experimental design

The animals were daily checked for spontaneous delivery of fetuses. The day when delivery was confirmed was designated as postnatal day (PND) 0 of the pups. The pups were collected and assigned to treatment groups on PND 1. The pups in various treatment groups were identified using tattoos and placed under the same dams at eight pups per dam. If the number of treated pups was less than eight, untreated male pups were included to adjust the litter size to eight. In the present study, eight foster dams nursed four to seven female pups comprising one or two pups of each treatment group.

The animals were injected subcutaneously using a microsyringe (80401, Hamilton, NV, USA) on PND 1 with 0.08, 0.4, or 2 μg/kg EE or with 1 ml/kg corn oil (vehicle control). BW of the pups was measured on PND 1, weekly from PND 7, and on the day of necropsy. During the nursing period, the age at eye opening was determined as an index of physical development. The pups were weaned on PND 21 and examined for vaginal opening from PND 28. BW of the rats showing vaginal opening was measured. Estrous cycles were determined by vaginal cytology for two weeks at two-week intervals from postnatal week (PNW) 8-33. Estrous cycles were categorized as regular cycle (4-5 day cycle), persistent estrus (no consecutive days judged as diestrus or metestrus), and irregular cycle (neither regular cycle nor persistent estrus). In addition, the number of days judged as estrus or proestrus and those judged as diestrus or metestrus were calculated for each period.

Necropsy was performed during PNW 33-34 on the day of estrus if possible. The animals were euthanized under sodium pentobarbital anesthesia (Somnopentyl; Schering-Plough Animal Health, Osaka, Japan) by bleeding from the abdominal aorta. The pituitary gland, mammary gland, and major thoracic and abdominal organs were dissected, then, the ovaries, uterus, thymus, liver, kidneys and adrenal glands were weighed. The ova-

ries were fixed in Bouin's solution, and mammary glands were fixed in phosphate-buffered 10% formalin. Oviducts were collected to determine ovulation using Burdick and Whitney's method (Burdick and Whitney, 1941). Blood was centrifuged at 2,500 rpm for 25 min at 4°C, and serum was stored at -50°C for further analyses. The fixed ovaries were embedded in paraffin wax according to standard procedures. The specimens were cut serially into sections of 5 μm in thickness and stained with hematoxylin and eosin. The fixed mammary glands were also embedded in paraffin wax by the same method, and were cut into sections of 5 μm in thickness and stained with hematoxylin and eosin.

Statistical analysis

Statistical analysis was performed using JMP Statistical Analysis Software (SAS Institute, Cary, NC, USA). All data were analyzed using one-way analysis of variance (ANOVA). Differences between the control and EE-treated groups were analyzed by Dunnett's test. A P-value < 0.05 was considered statistically significant.

RESULTS

Effects of EE on growth and puberty

The mean BW (± standard error of the mean, S.E.M.) on PND 1, the day of administration, in the 0, 0.08, 0.4, and 2 μg kg EE-treated groups was 7.0 (± 0.3), 7.4 (± 0.3), 7.5 (± 0.3), and 7.4 (± 0.3), respectively, and the mean dose of EE per animal in these groups was calculated to be 0, 0.59, 3.00, and 14.8 ng, respectively. As shown Fig. 1, there was no significant difference in the growth of animals among the groups, and other general abnormalities related to EE-treatment were not observed any group (data not shown). The timings of eye opening and vaginal opening of the treated animals were not significantly different between the EE-treated and control groups (Table 1).

Effects of EE on the estrous cycle

To determine if EE exposure had any effect on sexual development, we assessed pattern of the estrous cycle (Fig. 2) and the numbers of estrous cycles and days judged as estrus or proestrus (estrous/proestrous days) for each observation period (Table 2). In addition, the proportions of estrous/proestrous days and those judged as diestrus or metestrus (diestrous/metestrus days) during each observation period are illustrated in Fig. 3. In the control group, the numbers of estrous cycles and estrous/proestrous days for each observation period did not vary significantly during PNW 8-9 and rest of the periods, and no animal showed persistent estrus until PNW 33. In the EE-treated groups, the parameters were not significantly differ-

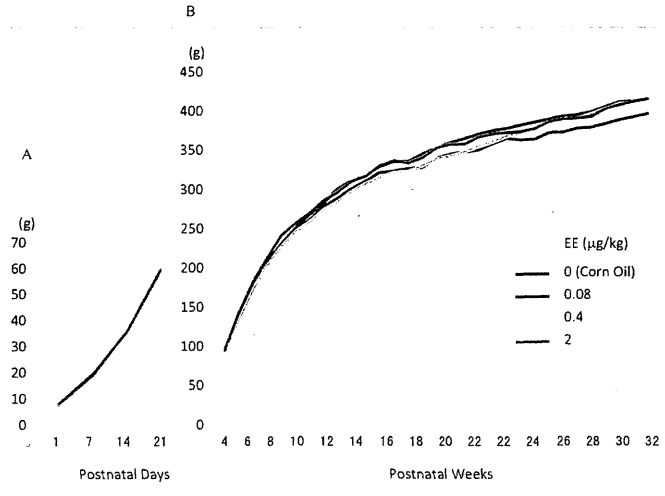

Fig. 1. Body weight changes of animals during the pre- (A) and post- (B) weaning periods. The animals were administered 0, 0.08, 0.4 or 2 μg kg of 17α-ethynylestradiol (EE) subcutaneously on postnatal day 1. N = 11 until postnatal week 4, and N = 8-9 from postnatal week 5 to 34.

Table 1. Effects of neonatal 17α-ethynylestradiol (EE) exposure on the body weight (BW), age at eye opening, and age at vaginal opening of female rats subcutaneously treated with EE on postnatal day 1.

Dose of EE (μg/kg)	0	0.08	0.4	2
Number of animals examined	11	10	11	11
Age at eyes opening (day)	13.8 ± 0.1	13.9 ± 0.2	13.7 ± 0.1	13.6 ± 0.1
BW at eyes opening (g)	35.1 ± 0.9	34.8 ± 0.9	35.4 ± 0.9	35.0 ± 0.9
Age at vaginal opening (day)	32.1 ± 0.5	33.0 ± 0.5	32.2 ± 0.5	32.5 ± 0.5
BW at vaginal opening (g)	115 ± 3	120 ± 3	118 ± 3	121 ± 3

Values represent mean ± standard error of the mean (S.E.M).

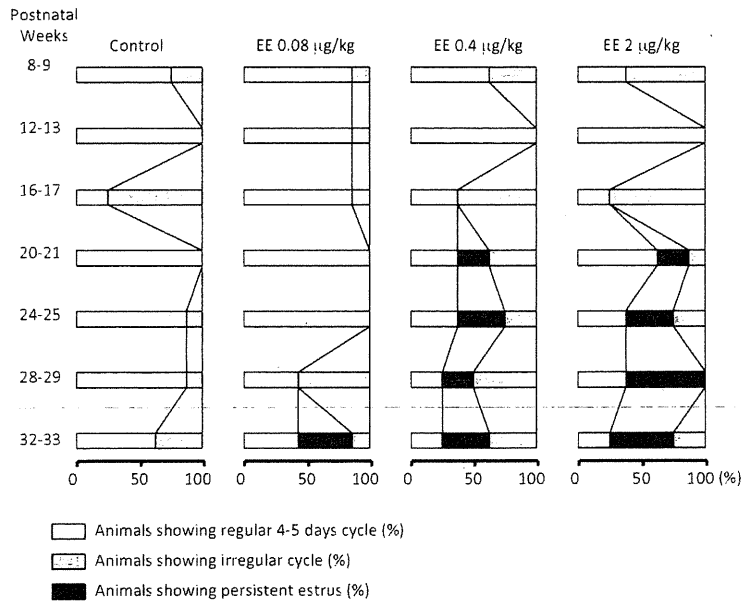

Fig. 2. Proportions of animals with regular (4-5 days) cycle, irregular cycle or persistent estrus during each observation period. EE, 17α-ethynylestradiol

ent compared with those in the control group until PNW 17, whereas the number of estrous cycles occurring during PNW 12-13 in the 2 μg/kg EE-treated group was significantly smaller than that in the control group (Table 2) because of normal but slightly longer intervals of the estrous cycle (mean ± S.E.M. was 4.4 days ± 0.1 in the 2 μg/kg EE-treated group and 4.0 days ± 0.1 in the control group); however, from PNW 20-21, the animals in the 0.4 μg/kg or more EE-treated groups began to show persistent estrus (Fig. 2), and the number of estrous cycles occurring during PNW 20-21 in the 2 μg/kg EE-treated group was significantly lesser than that in the control group (Table 2). In addition, the number of estrous proestrous days became significantly greater from PNW 24-25 in the 2 μg/kg EE-treated group and PNW 28-29 in the 0.4 μg/kg EE-treated group compared with that in the control group (Table 2, Fig. 3). Furthermore, the animals in the 0.08 μg/kg EE-treated group began to show persist-

Table 2. Effects of neonatal 17α-ethynylestradiol (EE) exposure on the number of estrous cycle revolved and the number of estrous proestrous days for each observation period in the female rats subcutaneously treated with EE on postnatal day 1.

Dose of EE (μg/kg)	0	0.08	0.4	2
Number of animals examined	8	7	8	8
Observation period (postnatal week)		The number of estrous cycle revolved		
8-9	1.9 ± 0.2	1.0 ± 0.2	1.5 ± 0.2	1.6 ± 0.2
12-13	2.4 ± 0.2	2.4 ± 0.2	2.3 ± 0.2	1.8 ± 0.2*
16-17	1.6 ± 0.2	2.0 ± 0.3	1.3 ± 0.2	1.4 ± 0.2
20-21	2.1 ± 0.3	2.4 ± 0.3	1.6 ± 0.3	1.1 ± 0.3*
24-25	2.0 ± 0.3	2.3 ± 0.3	1.5 ± 0.3	1.3 ± 0.3
28-29	1.8 ± 0.3	1.4 ± 0.4	1.3 ± 0.3	1.1 ± 0.3
32-33	1.6 ± 0.3	1.4 ± 0.4	0.8 ± 0.3	0.8 ± 0.3
		The number of estrous proestrous days		
8-9	5.0 ± 0.5	5.9 ± 0.6	5.5 ± 0.5	5.3 ± 0.5
12-13	6.0 ± 0.5	5.6 ± 0.6	6.0 ± 0.5	5.0 ± 0.5
16-17	6.0 ± 0.8	5.0 ± 0.8	6.0 ± 0.8	6.3 ± 0.8
20-21	4.5 ± 1.1	5.7 ± 1.2	7.0 ± 1.1	8.1 ± 1.1
24-25	4.5 ± 1.0	5.4 ± 1.1	8.1 ± 1.0	9.3 ± 1.0**
28-29	3.9 ± 1.2	7.0 ± 1.2	9.4 ± 1.2**	9.8 ± 1.2**
32-33	4.5 ± 1.5	8.6 ± 1.6	9.6 ± 1.5**	9.8 ± 1.5**

Values represent mean ±standard error of the mean (S.E.M.).
* and **, significantly different from control at $p < 0.05$ and 0.01 respectively.

ent estrus at PNW 32-33: however, significant differences were not observed in any parameter. Thus, the estrous cycle in the EE-treated groups had a marked tendency to lose cyclic revolution, and the animals had longer proestrus or estrus days.

Effects of EE on ovulation, organ weight, and macro- and histopathology

Subcutaneous spots or macular milk accumulations were macroscopically identified at necropsy in 0/8, 1/7, 4/8, and 3/8 animals in the 0, 0.08, 0.4 and 2 μg/kg EE-treated groups, respectively (Fig. 4A). Microscopical examination revealed these lesions in the 2 μg/kg EE-treated groups, and acinar cell hyperplasia with milk secretion and dilated ducts were found in the mammary gland (Fig. 4B); however any proliferating lesion including anterior adenoma or focal hyperplasia was not detected in the pituitary of the animals with mammary gland hyperplasia (data not shown).

There were no significant differences in the weights of non-reproductive organs between the control and EE-treated groups, although EE-treated animals may have slightly larger pituitary and adrenal glands (Table 3). The weights of ovaries and uteri were analyzed based on whether or not the animals had ovulated at the time of necropsy (Table 4). Except for one animal showing persistent estrus, all the control animals observed at estrus were confirmed to have ovulated and had shed a normal number of oocytes. Because only one animal in the 0.08 and 2 μg/kg EE-treated groups had ovulated, we only compared the weights of ovaries and uteri of the EE-treated animals with those of the unovulated control animals and found no significant differences in the organ weight. Corpus luteum was observed in approximately half of the EE-treated animals (Table 5); however, the majority contained cystic follicles (Fig. 5, Table 5). Among the animals lacking corpus luteum, two and one animals in the 0.4 and 2 μg/kg EE-treated groups, respectively, showed irregular estrous cycle until necropsy, whereas the others showed persistent estrus.

DISCUSSION

The perinatal period is critical for brain sexual dif-

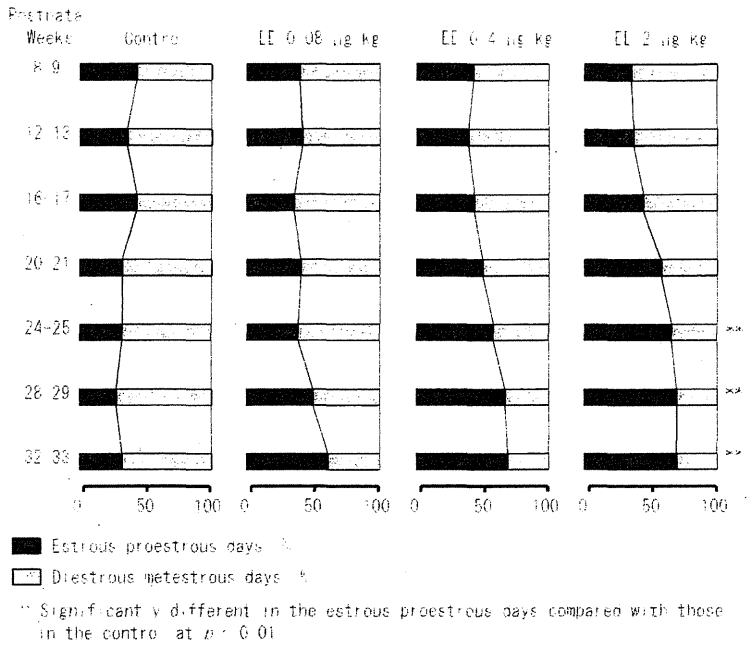

Fig. 3. Proportions of days judged as estrus or proestrus (estrous/proestrous days) and those judged as diestrus or metestrus (diestrous/metestrus days) during each observation period. EE, 17α-ethynylestradiol.

Table 3. Effects of neonatal 17α-ethynylestradiol (EE) exposure on weights of non-reproductive organs collected from female rats subcutaneously treated with EE on postnatal day 1.

Dose of EE (μg/kg)	0	0.08	0.4	2
Number of animals examined	8	7	8	8
BW at necropsy (g)	395 ± 19	399 ± 20	422 ± 19	414 ± 19
Pituitary	19 ± 2	22 ± 2	26 ± 2	24 ± 2
Thymus (mg)	148 ± 13	145 ± 14	133 ± 13	161 ± 13
Liver (g)	11.9 ± 0.6	12.1 ± 0.7	13.2 ± 0.6	12.7 ± 0.6
Kidneys (g)	2.2 ± 0.1	2.2 ± 0.1	2.3 ± 0.1	2.4 ± 0.1
Spleen (mg)	611 ± 27	636 ± 29	633 ± 27	641 ± 27
Adrenal glands (mg)	72 ± 4	80 ± 5	82 ± 4	84 ± 4

Values represent mean ± standard error of the mean (S.E.M.).

ferentiation in rodents and is highly sensitive to aromatizable testosterone or estrogenic compounds, including synthetic estrogens, xenoestrogens, and phytoestrogens. Aromatized estrogenic compounds inappropriately masculinize the brain in female neonates and lose cyclic revolution of estrus after puberty (reviewed in Gore, 2008). In the present study, we have shown that neonatal exposure to EE interfered with the estrous cycle, by decreasing the number of estrous cycle revolved and causing persistent estrus, whereas untreated animals had normal

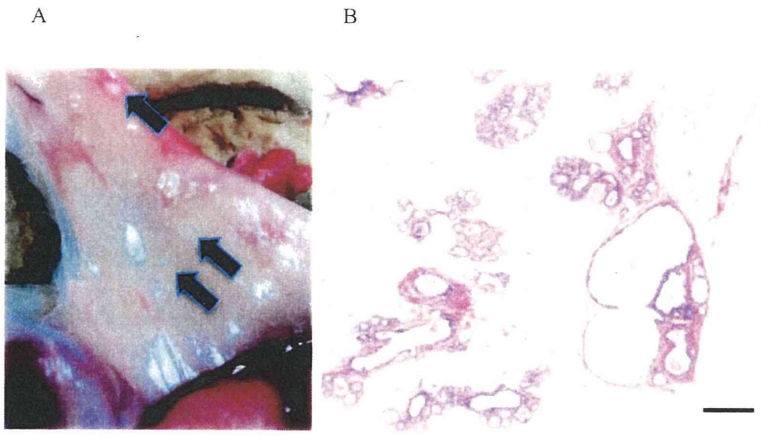

Fig. 4. A representative photograph found macular accumulations of milky secretion (arrows in A) and a histopathological appearance of the mammary gland (B) collected at the terminal necropsy from female rats exposed to 2 μg/kg of 17α-ethynylestradiol on postnatal day 1. Note acinar cell hyperplasia with milk secretion and duct dilatation in the mammary tissue. (Bar = 200 μm)

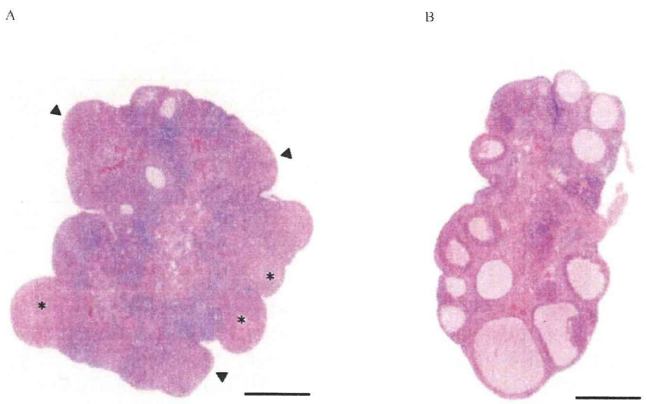

Fig. 5. Histological appearance of the ovaries collected at the terminal necropsy from female rats exposed to 0 (A) or 2 μg/kg of 17α-ethynylestradiol (B) on postnatal day 1. Note that there are newly formed (arrowheads) and morphologically regressing corpora lutea (asterisks) in the control ovary (A). In the 17α-ethynylestradiol–treated ovary, there are large cystic or atretic follicles but no corpus luteum (B). (Bars = 1 mm)

4–5 day estrous cycles. Thus, single neonatal exposure to 0.4 μg/kg EE had adverse effects more than 20 weeks later, highlighting the vulnerability of brain sexual differentiation to exogenous estrogenic compounds during this critical period (Morris *et al.*, 2004; Gore, 2001).

This effect was also dose-dependent, occurring the earliest in the animals treated with the highest dose, although the Sprague-Dawley rat strain is known to develop persistent vaginal cornification with age (Goldman *et al.*, 1986; Suzuki *et al.*, 2004). Some animals in the 0.08 μg/kg EE-treated group

Table 4. Weights of reproductive organs from female rats treated with 17α-ethynylestradiol (EE) subcutaneously on postnatal day 1.

Dose of EE (μg/kg)	0	0.08	0.4	2
Animals examined	8	7	8	8
Animals on the day of estrus*	6	7	8	8
Animals found ovulating	5	1	0	1
Body weight (g)	377 ± 21	409		453
Number of oocytes shed	13.2 ± 1.2	1		13
Ovaries (mg)	94 ± 6	113		137
Uterus (mg)	630 ± 37	614		636
Presence of cystic follicles	0	0		1
Animals not ovulating	3	6	8	7
Body weight (g)	425 ± 34	397 ± 24	422 ± 21	411 ± 24
Ovaries (mg)	82 ± 10	59 ± 7	61 ± 6	61 ± 7
Uterus (mg)	606 ± 77	780 ± 54	664 ± 47	700 ± 54

Values represent mean ± standard error of the mean (S.E.M.).
*Including animals showing persistent estrus.

Table 5. Histological evaluation of ovaries collected at terminal necropsy from female rats subcutaneously treated with 17α-ethynylestradiol (EE) on postnatal day 1.

Dose of EE (μg/kg)	0	0.08	0.4	2
Animals examined	8	7	8	8
Absence of corpus luteum	1	3	5	4
Presence of cystic follicles	2	5	5	7

began to show persistent estrus at PNW 32-33; however, no statistical differences were observed. Considering the dose-related increase in the frequency of persistent estrus in the 0.4 μg/kg or more EE-treated group, occurrence of the persistent estrus in the 0.08 μg/kg treated group might reflect both effects of neonatal EE treatment and spontaneous aging. Thus, such effects of a lower dose of estrogenic compounds could be hidden by aging.

Although EE is a common synthetic estrogen used for various medical purposes, its effects on female reproductive development have been studied only by administration to pregnant or lactating dams (Tinwell *et al*., 2002; Fusani *et al*., 2007; Ryan *et al*., 2010). Oral treatment of pregnant dams during gestational days 6-21 accelerated vaginal opening of their offspring and increased period in the estrus at a dose level of 20 μg/kg/day (Tinwell *et al*., 2002). Similarly, oral treatment of pregnant and lactating dams with 5 μg/kg/day EE from gestational day 7 to PND 18 has been reported to cause abnormalities such as development of cleft phallus, acceleration of vaginal opening and reduction of fecundity (Ryan *et al*., 2010). In contrast, 0.4 ng/kg/day of EE did not have any effect on reproductive development of female offspring from dams orally treated with EE from gestational day 15 to PND 21 (Fusani *et al*., 2007). Except this dose level, transgenerational administration with various doses of EE seems to affect the estrous cycle; however, the present study revealed that single subcutaneous injection with at least 0.08 μg/kg of EE to pups at PND 1 affects estrous cycle at a later period.

Because *in vivo* administration with estrogenic compounds is able to increase uterine weight of ovariectomized or immature intact female rats or mice, the "rat uterotropic bioassay", has been established for evaluation of estrogenic potency of chemical substances *in vivo* (Kanno *et al*., 2001). In the phase 1 validation study for the bioassay (Kanno, *et al*., 2001), where the immature and adult ovariectomized rat uterotrophic assays were performed in 19 laboratories with a reference agonist of estrogen receptors, EE, the lowest observed effect level

(LOEL) of EE required to increase the uterine weight in immature and ovariectomized adult rats was reported to be 0.03-0.3 and 0.1-1.0 μg/kg/day, respectively, for three days by subcutaneous injection. The lowest dose applied in the present single injection study is similar to or lower than these levels. Based on our data, we conclude that the estrous cycle is one of sensitive biomarkers to evaluate delayed effects of neonatal exposure to estrogenic compounds.

In the present study, the lowest dose of EE took the longest time to cause persistent estrus, suggesting the possible involvement of a dose-dependent mechanism in acceleration of reproductive senescence. Exposure of neonatal female rats to 0.1-100 μg of estradiol benzoate (EB) led to a dose-dependent decrease in hypothalamic *Kiss1* mRNA levels during the prepubertal period (Navarro *et al.*, 2009). *Kiss1* encodes a family of kisspeptin neuropeptides, which are critical upstream regulators of gonadotropin-releasing hormone (GnRH) secretion. Kisspeptin signaling is essential for the onset of puberty and control of preovulatory gonadotropin surges (Adachi *et al.*, 2007; Tena-Sempere, 2010; Roa *et al.*, 2011). Perinatal exposure to various estrogenic compounds, including genestein (Losa *et al.*, 2011) and bisphenol-A (Patisaul *et al.*, 2009), disrupt the kisspeptin system in the anteroventral periventricular nucleus (AVPN), where the GnRH surge is generated. Furthermore, exposure of the hypothalamus of adult female rats to 1 mg of EB or 10 μg or 100 mg of methoxychlor during the prenatal and postnatal periods caused up-regulation of *Kiss1* and down-regulation of *estrogen receptor α* (*Esr1*) in the preoptic area and also caused extensive methylation of the *Esr1* promoter (Gore *et al.*, 2011). Based on these findings, similar dose-dependent alterations might also occur following single exposure to low-dose EE used in our study, average: 0.59-14 ng.

In addition to reproductive senescence, leakage of milk from the mammary gland was frequently observed in the EE-treated animals. Because hyperplasia of the mammary gland was histologically evident, milk production is likely to be enhanced in nulliparous rats in the present study. While the milk was found in most animals showing persistent estrus, mammary hyperplasia was not always explained by the endocrinological imbalance caused by reproductive senescence. There is abundant evidence that neonatal exposure to estrogenic compounds such as diethylstilbestrol (Umekita *et al.*, 2011), BPA (Betancourt *et al.*, 2010; Ayyanan *et al.*, 2011), and butyl benzyl phthalate (Moral *et al.*, 2011) can directly cause morphological or molecular alterations in the mammary gland. Detailed examination, including quantification and analysis of methylation for genes encoding such molecules, may help to understand the causes of milk production in nulliparous female rats.

In the present study, we observed no discernible effect of EE exposure on the timing of vaginal opening. There are conflicting reports on the effect of perinatal exposure to estrogenic compounds on the timing of vaginal opening. Perinatal exposure to butyl benzyl phthalate (Moral *et al.*, 2011) or *p*-octylphenol (Nagao *et al.*, 2001) modify the timing of vaginal opening, whereas neonatal exposure to bisphenol A, nonylphenol or genistein have a negative effect on the timing of vaginal opening (Noda *et al.*, 2005).

Although ovulation was detected in all the control animals examined on the day of estrus, the majority of EE-treated animals, even those with an irregular estrous cycle, had not ovulated. Furthermore, several animals in the EE-treated groups and one in the control group lacked corpus luteum. In the ovaries of cycling rats, there are different generations of corpora lutea, because several estrous cycles are required for complete elimination of corpora lutea by structural regression. Therefore, the animals lacking corpus luteum are judged to be anovulatory in the long term.

In conclusion, single exposure to low-dose EE during the critical period of brain sexual differentiation causes delayed effects on the estrous cycle and the mammary gland, and these effects could be hidden by spontaneous aging in rats.

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Vascular Hamartoma in the Uterus of a Female Sprague-Dawley Rat with an Episode of Vaginal Bleeding

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Abstract

An annular, reddened lesion with mild serosal hemorrhage and no tumorous mass formation was detected in the right uterine horn of a 37-week-old female Sprague-Dawley rat that had postpubertal vaginal bleeding. Histological examination revealed prominent proliferation of the endometrium, which occupied the uterine lumen. There were numerous aberrant vascular spaces filled with erythrocytes, proliferation of stromal cells, and inflammatory infiltrates including hemosiderin-laden macrophages in the endometrium. These vasculatures extended into the myometrium, and in a transverse section of the lesion, they were mostly distributed throughout the circumference of the uterus. They were irregular in shape and interconnected, forming a large vascular sinus and anastomosing reticular channels. In the area with serosal hemorrhage, the muscular layer covering the large irregular vascular space had undergone degeneration and necrosis. The lining cells of the vasculatures were often plump, and they protruded into the lumen and were arranged in a tombstone or hobnail manner. Immunostaining revealed that these cells were positive for von Willebrand factor and CD34. The aberrant vasculatures were not accompanied by pericytes or muscular layer, although a discontinuous muscular wall was present around some of them. From these results, the uterine lesion was diagnosed as a vascular hamartoma.

Keywords: histopathology; rat pathology; reproductive system; vascular system.

Introduction

Spontaneous vascular tumors are not common in laboratory rats, although hemangiomas, hemangiosarcomas, hemangiopericytomas, and lymphangiosarcomas have been detected in aged control rats in carcinogenicity studies. The first 2 of these tumors have been found in the spleen, lymph nodes, liver, skin, heart, blood vessels, abdominal cavity, cranial cavity, thoracic cavity, eye, kidney, testis, vagina, and uterus (Baldrick 2005; Brix et al. 2005; Chandra, Riley, and Johnson 1992; Dinse et al. 2010; Goodman et al. 1979; Keenan et al. 1995; McMartin et al. 1992; Nakazawa et al. 2001; Zwicker et al. 1995). Among these organs, the spleen, skin, and lymph nodes are the most common sites of these tumors. The overall incidence of the hemangiomas and hemangiosarcomas in control animals in 2-year carcinogenicity tests with Sprague-Dawley (SD) rats are 0.19 to 0.5% and 0.3 to 0.9%, respectively (Brix et al. 2005; Chandra, Riley, and Johnson 1992; Dinse et al. 2010; McMartin et al. 1992; Zwicker et al. 1995). With regard to the age of rats with these tumors, Zwicker et al. (1995) observed 8 cases of hemangiomas in rats with an average age of 658 days (range, 565–735) and 5 cases of hemangiosarcomas in rats with an average age of 685 days (range, 638–728) in SD rats. In a report by McMartin et al. (1992), hemangiopericytomas occurred in 0.2% of females and 0% of males, and lymphangiosarcomas occurred in 0.2% of males and 0% of females of aged control SD rats (585 of each sex).

Vascular uterine tumors have been rarely reported in aged SD rats, and none have been recorded in Fischer 344 rats (Dinse et al. 2010; Goodman et al. 1979) or Wistar rats (Poteracki and Walsh 1998). A uterine hemangioma was found in 1 of the 710 female SD rats (Zwicker et al. 1995) and 1 of the 350 female SD rats (Keenan et al. 1995). However, the incidence of uterine hemangiosarcomas has been reported to be 0.08% of 1,329 female SD rats (Chandra, Riley, and Johnson 1992). In humans, vascular uterine tumors are very rare, and they may cause uterine bleeding (Sharma et al. 2006; Virk, Zhong, and Lu 2009; Weissman, Talmon, and Jakobi 1993).

In this article, we describe the histological features and have discussed the nature of the aberrant and localized vascular proliferative lesions that were detected in the uterine horn of an adult female SD rat that experienced a postpubertal episode of vaginal bleeding.

Case Report

The animal was a 37-week-old female SD rat (CrJ:CD; Charles River Japan Inc., Kanagawa, Japan) that received a single subcutaneous administration of 7 µl of 17α-ethynyl estradiol (EE, Sigma-Aldrich Corporation, St. Louis, MO) that was concentrated in 0.08 µg/ml 1 day after birth; 4 other littermates received a similar administration. The rats were housed

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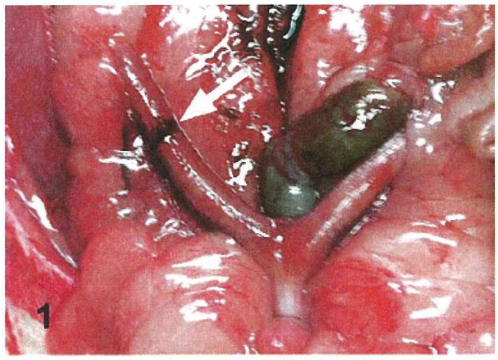
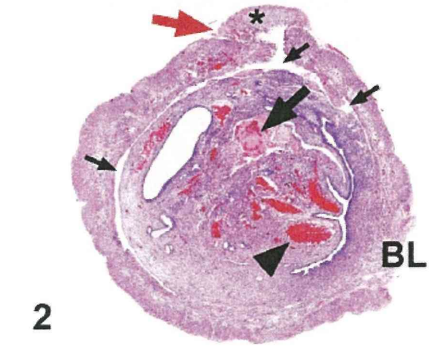
Abbreviations: α-SMA, α-smooth muscle actin; H&E, hematoxylin and eosin; PCNA, proliferating cell nuclear antigen; SD, Sprague-Dawley; vWF, von Willebrand factor.





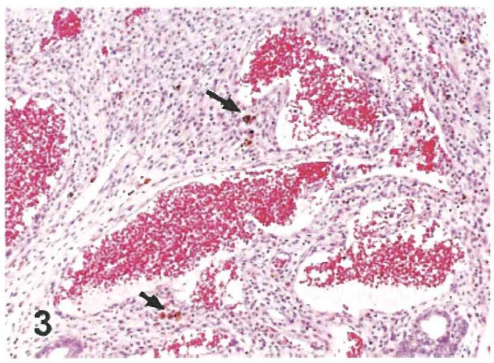


FIGURE 1.—Macroscopic feature of the lesion in the right uterine horn (white arrow). FIGURE 2.—Low-power views of the microscopic features of the uterine lesion. Endometrial proliferation occludes the uterine lumen. There are many cavernous vascular spaces filled with red blood cells (arrow head). The large arrow indicates a thrombus in the irregularly dilated vascular lumen. Long, irregular vascular spaces along the muscular layers cover two-third of the uterine circumference on a transverse section (small arrows). The asterisk indicates degeneration of uterine wall. A higher magnification image of the lesion indicated by the red arrow is shown in Figure 5. H&E (1×).

in a barrier system animal facility with a 12-hr light/dark cycle, a temperature of 21 ± 1°C, and a relative humidity of 50 to 60%. The rats were fed a CLEA Rodent Diet CE-2 (CLEA Japan, Inc., Tokyo, Japan), and they had free access to tap water. A 14-day evaluation of vaginal smears was performed every 4 weeks from 8 weeks of age. At 28 weeks of age, vaginal bleeding was detected in one of the rats. Therefore, smear sampling was stopped, and bleeding was observed until 32 weeks of age. Vaginal smear evaluation revealed recurrent estrus that occurred in 4- or 5-day cycles until the start of bleeding. Smear sampling was restarted at 35 weeks of age, and no bleeding was observed for 2 weeks. At 37 weeks of age, the rat was deeply anesthetized with sodium pentobarbital (Somnopentil, Kyoritsu Shoji Co., Tokyo, Japan) and euthanized by postcava bleeding. All procedures in this study were performed in accordance with the guidelines approved by the Animal Research Committee of our institution.

At necropsy, the body weight was 554 g, and no external abnormalities were observed. However, an annular reddened lesion 3 mm wide with a slight serosal hemorrhage was found in the right uterine horn with no mass formation (Figure 1). There were no gross abnormalities in any other organs. The systemic tissues, including the uterus, were excised, fixed in 10% phosphate-buffered neutral formalin, and embedded in paraffin according to a routine method. Serial paraffin sections were prepared and stained with hematoxylin and eosin (H&E); some sections were used for immunostaining. In addition, sections from other systemic organs were stained with H&E for light microscopy. Immunostaining was performed with an immunoenzyme polymer method with goat anti-rat CD34 (R&D Systems, Inc., Minneapolis, MN), rabbit anti-human von Willebrand factor (vWF) antibody (DAKO Denmark A/S, Glostrup, Denmark), mouse anti-human α-smooth muscle actin (α-SMA) antibody (clone 1A4; DAKO Denmark A/S), and mouse antiproliferating cell nuclear antigen (PCNA) antibody (clone PC10; DAKO Denmark A/S) as primary antibodies. The immunoreaction was visualized by peroxidase-conjugated anti-goat immunoglobulin (Ig; Histofine Simple Stain MAX-PO (G); Nichirei Biosciences Inc., Tokyo, Japan), peroxidase-conjugated anti-rabbit Ig (Histofine Simple Stain MAX-PO (R); Nichirei Biosciences Inc.) or peroxidase-conjugated anti-mouse Ig (Histofine Simple Stain MAX-PO (M); Nichirei Biosciences Inc.), and 3,3′-diaminobenzidine. In all immunostainings, Mayer's hematoxylin was used for counterstaining.

Light microscopic examination of the uterine lesion revealed prominent proliferation of the endometrium, which mostly occupied the uterine lumen. There were many aberrant cavernous vascular spaces filled with erythrocytes, occasionally accompanied by thrombi (Figure 2). In addition, diffuse proliferation of spindle stromal cells and infiltration of eosinophils, neutrophils, lymphocytes, and hemosiderin-laden

FIGURE 2. (Continued). BL = broad ligament of the uterus. FIGURE 3.—Cavernous vascular spaces in the endometrium with intense stromal cell proliferation and cellular infiltration, including hemosiderin-laden macrophages (arrows). H&E (100×).

macrophages were observed in the endometrium (Figure 3). Necrosis and desquamation of the endometrial epithelium were not found. The irregular vascular spaces extended from the endometrium to the myometrium and were distributed over nearly two-third of the uterine circumference on a transverse section, as shown in Figure 2. Examination of the serial sections revealed that these vasculatures were prominently irregular in shape and interconnected, and they formed a large vascular sinus or anastomosing reticular vascular channels (Figure 2). In the area with serosal hemorrhage (Figure 2), the muscular layer covering the large irregular vascular space had undergone degeneration and necrosis with proliferation of fibroblasts and infiltration of macrophages, resulting in perforation of the uterine wall and serosal hemorrhage (Figures 4 and 5). The lining cells of the endometrial cavernous spaces were mostly flat (Figure 3), while those of other aberrant vasculatures found throughout the uterine wall were often cuboidal or plump. They protruded into the lumen and were arranged in a tombstone or hobnail manner (Figure 6). In some lumens, the lining cells were plump and protruded into the lumens, but they were flat as those in the normal vessels on the other side (Figure 7). Mitosis was not detected in the flat endothelial cells and was rarely found in the plump lining cells.

Immunohistochemical examination revealed that the lining cells of the aberrant vasculatures were positive for vWF (Figure 8A) and CD34 (Figure 8B). However, immunoreactivity for vWF was less intense than that in normal endothelial cells. Staining for PCNA was frequently positive in the lining cells of the aberrant vasculatures (Figure 8C), whereas normal endothelial cells were positive only occasionally. In addition, endometrial epithelium and stromal cells, myometrial cells, and fibroblasts were frequently positive for PCNA. Normal blood vessels in the lesion were encircled by α-SMA-positive pericytes or smooth muscle layers. The aberrant vasculatures were not accompanied by α-SMA-positive pericytes or muscular layer, although discontinuous muscular walls had formed around some abnormal vessels (Figure 9).

The aberrant vasculatures were localized to the portion of the uterine horn that corresponded to the gross annular lesion. No abnormalities were found in any of the other uterine tissues, including the opposite uterine horn, ovaries, or other systemic organs. The littermates of the experimental rat did not show any lesions in their main organs, including the uterus.

The results of immunohistochemical examination suggested that the lining cells of the aberrant vasculatures in the uterine lesion were vascular endothelial cells with high proliferative activity. In rats, an endometrial stromal polyp, which is often highly vascular throughout the stroma, is sometimes termed an angiomatous polyp (Goodman and Hildebrandt 1987). A cavernous hemangiomatous polyp (Sharma et al. 2006) is accompanied by many neoplastic vasculatures in the endometrium. However, the structure and distribution of the aberrant vasculatures observed in the present case were different from those previously recorded in human and rat uterine tumors (Dixon et al. 1999).

The present case and its littermates were administered 17α-ethynyl estradiol 1 day after birth. Estrogen induces vascular endothelial growth factor in the uterus, which promotes vascular growth and increases vascular permeability (Cullinan-Bove and Koos 1993). However, no uterine vascular lesions were found in the littermates of the present case, and those rats in the other experiment performed by our group received doses of 17α-ethynyl estradiol that were the same or higher (Shirota et al. 2012). In addition, the lesion was localized to a limited portion of the uterine horn. These findings obviously indicated that there were no close relationship between the 17α-ethynyl estradiol administered and the development of the uterine lesion.

In general, neoplastic vasculatures form massive or nodular lesions in both benign and malignant tumors. In contrast, the uterine lesion in this case did not form a tumorous mass. The aberrant vasculatures showed cavernous, sinusoidal, or incomplete venous appearances. They extended throughout the uterine wall and expanded to nearly the entire circumference of the affected site of the uterine horn, but they were unlikely to have been invasive, destructive, or compressive. In addition, cavernous or sinusoidal structures were not associated with α-SMA-positive pericytes, and some vasculatures had discontinuous muscular walls. Heterogeneity of the vasculatures composing a lesion is observed in some vascular hamartomas (Sugiyama et al. 2007; Yasuno et al. 2011) or congenital vascular abnormalities (Booler 2008; Redondo 2007), but not in vascular tumors (Calonje and Flecher 2007). As per these histological features of the uterine vasculatures, the lesion in the present case might be a vascular malformation rather than a true neoplasia.

The term vascular hamartoma, which has preferentially been used in veterinary pathology, is used as a synonym of vascular malformation in human pathology (Calonje and Flecher 2007). Human vascular malformations are benign non-neoplastic lesions, which are present from birth and often grow slowly during a lifetime (Redondo 2007). Most vascular tumors in rats have been found in aged control animals from 2-year carcinogenicity studies (Brix et al. 2005; Keenan et al. 1995). In addition, no vascular tumors have been detected in these studies in rats aged about 22 to 60 weeks by interim necropsy. The present case was 37 weeks of age, and fairly young compared with the rats with vascular tumors. Accordingly, the vascular lesion in the uterus was finally diagnosed as a vascular hamartoma. Expression of PCNA, which is frequently observed in nonendothelial cells in the uterus, might not be related to the vascular abnormalities, but might instead reflect the physiological conditions in the estrous cyclicity of the rat. The rat had a postpubertal episode of vaginal hemorrhage, which might have been related to the vascular lesion of the uterus.

There have been several reports of vascular hamartoma in animals (Calonje and Flecher 2007), and these studies illustrated the diverse morphology of vascular lesions in dogs (Beccaglia et al. 2008; Booler 2008; Corzo-Menendez et al. 2011; Smith and Van Winkle 2001; Yasuno et al. 2011), cattle (Benoit et al. 2005; Sugiyama et al. 2007), and a cat (Parkes et al. 2009). This report might be the first of a vascular hamartoma in a rat.

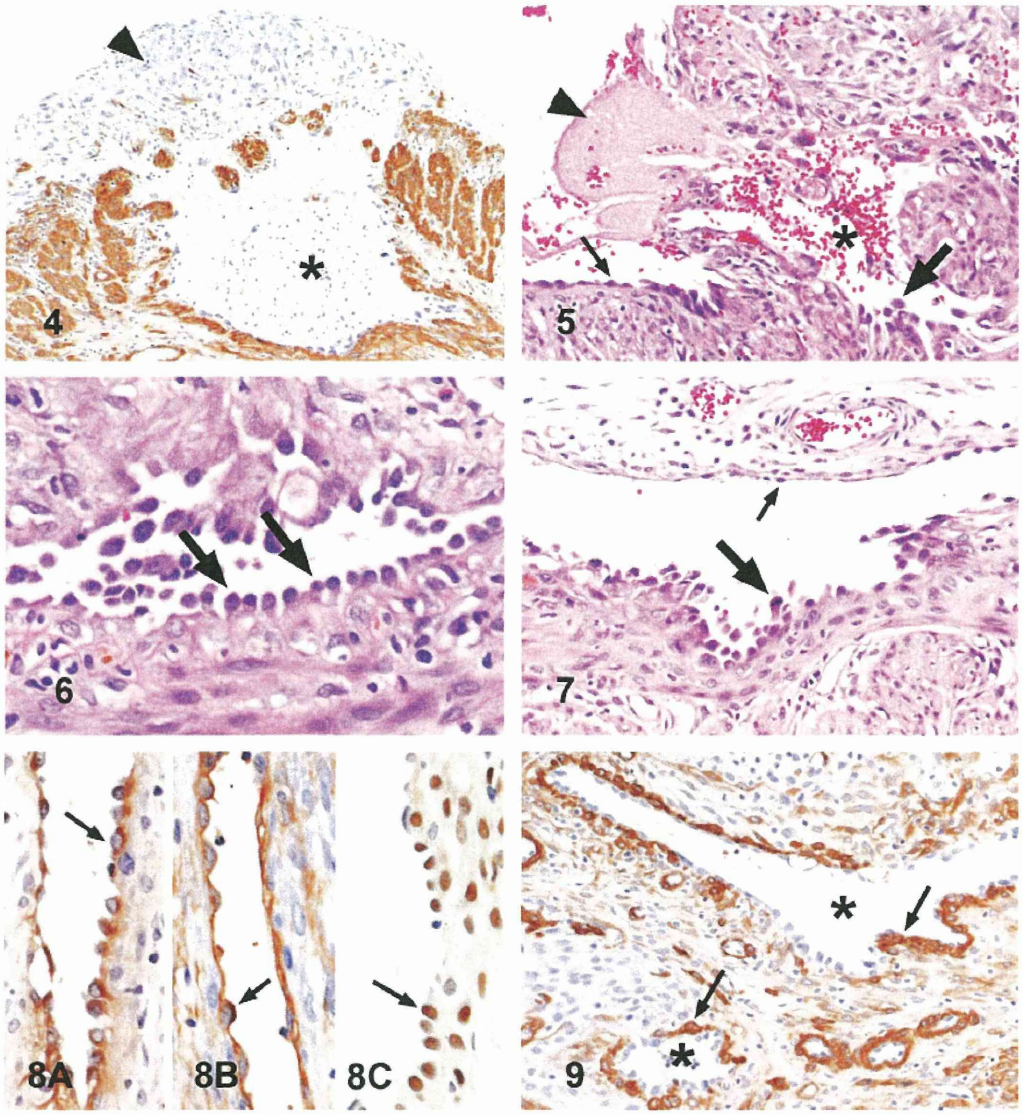


FIGURE 4.—Degeneration of the outer muscular layer covering a large, aberrant vascular space (arrowhead). Asterisk indicates a thrombus in the vascular space. Immunostaining for α-SMA. (100×). FIGURE 5.—High-power view of the ruptured portion of the uterine wall, as indicated by the framed rectangle in Figure 2. Aggregated fibrin is attached to the ruptured site (arrowhead). The vascular space (asterisk) is lined by endothelial cells (large arrow). The small arrow shows the serosal surface of the uterus. H&E (200×). FIGURE 6.—In the myometrium, plump lining cells protrude into the aberrant vascular lumens and are arrange in tombstone or hobnail manner (arrows). H&E (400×). FIGURE 7.—Aberrant vascular lumen lined by characteristic large endothelial cells protruding into the lumen (large arrow). The other side of the lumen is lined by small, flat endothelial cells (small arrow). H&E (200×). FIGURE 8.—Immunostaining for von Willebrand factor (A), CD 34 (B), and proliferating cell nuclear antigen (C) in the lining cells of aberrant vasculatures. A positive reaction is colored brown. (400×). FIGURE 9.—Immunostaining for α-SMA. Discontinuous muscular walls (arrows) are present around some abnormal vessels (asterisks). (200×).

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Thickened area of external granular layer and Ki-67 positive focus are early events of medulloblastoma in *Ptch1*$^{+/-}$ mice

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ABSTRACT

Patched1 (Ptch1) encodes a receptor for Sonic hedgehog (Shh) and is major gene related to human medulloblastoma (MB) in the Shh subgroup. MB is thought to arise from residual granule cell precursors (GCPs) located in the external granular layer (EGL) of the developing cerebellum. As the detailed preneoplastic changes of MB remain obscure, we immunohistochemically clarified the derived cell, early events of MBs, and the cerebellar developmental processes of *Ptch1*$^{+/-}$ (Ptch1) mice, an animal model of human MB of the Shh subgroup. In Ptch1 mice, the earliest proliferative lesions were detected at PND10 as focal thickened areas of outer layer of the EGL. This area was composed of GCP-like cells with atypia and nuclei disarrangement. In the latter cerebellar developmental period, GCP-like cell foci were detected at high incidence in the outermost area of the cerebellum. Their localization and morphological similarities indicated that the foci were derived from GCPs in the EGL. There were two types of the foci. A Ki-67-positive focus was found in Ptch1 mice only. This type resembled the GCPs in the outer layer of EGL characterized by having proliferating activity and a lack of neuronal differentiation. Another type of focus, Ki-67-negative, was observed in both genotypes and exhibited many of the same features of mature internal granule cells, suggesting that the focus had no preneoplastic potential. Due to morphological, immunohistochemical characteristics, our results indicate that the focal thickened area of EGL and Ki-67-positive foci are preneoplastic lesions of MB.

1. Introduction

Medulloblastoma (MB) is the most common malignant tumor in children which shows tremendous biological and clinical heterogeneity (Dhall, 2009; Hatten and Roussel, 2011; Jones et al., 2012). MB in humans is classified into four subtypes with distinct clinical, biological, and genetic profiles (Aref et al., 2012; Ellison et al., 2011; Jones et al., 2012; Kool et al., 2012; Mohan et al., 2012; Northcott et al., 2011). Molecular analysis of Sonic hedgehog (Shh) tumors in humans revealed activation of the Shh signaling pathway due to the loss of *Patched1 (Ptch1)* and mutations in other components of the Shh pathway. Approximately as high as 30% of MBs have mutations in Shh pathway components (Bhatia et al., 2012; Crawford et al., 2007; Klesse and Bowers, 2010; Oliver et al., 2005; Roussel and Hatten, 2011; Wang et al., 2012). *Ptch1* encodes a receptor for Shh, Patched1 (Ptch1), and is one of the major genes related to MB formation in humans (Dhall, 2009; Raffel, 2004). A subset of MBs has been identified with allelic loss of chromosome 9q22, a region that contains *Ptch1* (Dhall, 2009; Raffel, 2004). Pathway activation is triggered by binding of Shh to Ptch1, which in the absence of Shh suppresses the activity of Smoothened (Smo). Shh binding to Ptch1 or mutational inactivation of Ptch1 relieves the inhibition of Smo culminating in the activation of one or more of the Gli1 transcription factors that regulate the expression of downstream targets (Huse and Holland, 2010; Roussel and Hatten, 2011). Inappropriate activation of the Shh pathway is accepted as a cause of familial cancer due to inherited mutation of the *Ptch1* gene, which has been identified as responsible for nevoid basal cell carcinoma syndrome (Dhall, 2009; Klesse and Bowers, 2010).

Heterozygous *Ptch1* knockout mice (Ptch1 mice) display many of the typical symptoms of nevoid basal cell carcinoma syndrome, also known as Gorlin syndrome, including skeletal abnormalities, neural tube closure defects, a generalized over-growth, and predisposition to tumor formation (Corcoran and Scott, 2001; Hahn et al., 1999; Raffel, 2004). In addition, the Ptch1 mouse strain displays a high yield (14% up to 30%) of MB that resembles human

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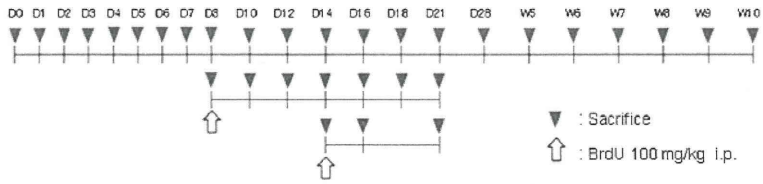

Fig. 1. Experimental design. Schedules for necropsy and the administration of BrdU are illustrated. Each time point represents at least 2 wild-type mice and 6 Ptch1 mice from over 2 dams. D, postnatal day; W, postnatal week.

MB of the Shh subgroup (Goodrich et al., 1997; Lau et al., 2012; Wetmore et al., 2000). In the mice, homozygous loss of *Ptch1* results in embryonic lethality at 9.5–10.5 days after fertilization (Goodrich et al., 1997). Thus, heterozygous *Ptch1* knockout mice have been used as a model for nevoid basal cell carcinoma/Gorlin syndrome including human MB, rhabdomyosarcoma, and basal cell carcinoma (Corcoran and Scott, 2001; Dyer, 2004; Hahn et al., 1999; Pazzaglia, 2006; Wu et al., 2011). Although Ptch1 mice are a valuable model for evaluation of drug efficacy and modulating effects of additional gene mutations, chemicals, or irradiation on brain tumor formation in childhood, the long latent period of 9 to over 12 months for assessment results in clinical signs of increased intracranial pressure (ataxia, decreased movement, paresis of hind limbs, enlarged occipital prominence, hunched back, and/or poor grooming) and death (Ayrault et al., 2009; Briggs et al., 2008; Ecke et al., 2009; Farioli-Vecchioli et al., 2007; Kimura et al., 2005; Pazzaglia et al., 2006, 2009; Pogoriler et al., 2006; Takahashi et al., 2012; Uziel et al., 2005; Wetmore et al., 2001). Therefore, detection of early indicators of MBs such as preneoplastic lesions in Ptch1 mice and evaluation with changes as an indicator of MB in short-term studies is needed.

To find early indicators of tumors in childhood, detailed investigation of normal developmental processes of target organs can be useful. Human MBs are thought to be derived from residual granule cell precursors (GCPs) located in the external granule cell or external granular (germinal) layer (EGL) of the cerebellum, although GCPs migrate inward to form the internal granule cell or internal granular layer (IGL) during normal cerebellar development (Behesti and Marino, 2009; Haldipur et al., 2012; Roussel and Hatten, 2011). The processes of cerebellar and MB development in Ptch1 mice are not well-defined.

This study was conducted to clarify the derived cell and early events of MBs, and cerebellar developmental processes in Ptch1 mice. We examined cerebella of Ptch1 mice and wild-type littermates sequentially during postnatal day (PND) 0 to 10 weeks of age.

2. Materials and methods

2.1. Animals

Ptch1 heterozygous knockout mice, generated by replacing exon 1 and 2 of the ptch1 gene with a LacZ/neomycin cassette (Goodrich et al., 1997), were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained in our laboratory. They were housed in polycarbonate cages with wood chip bedding and maintained in an air-conditioned animal room (temperature 24 ± 1 °C, relative humidity 55 ± 5%, 12-h light–dark cycle) with basal diet (CRF-1, Oriental Yeast Co., Tokyo, Japan) and tap water available *ad libitum*. The experimental protocol using animals was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

2.2. Necropsy

To examine following morphologic analysis necropsy was performed according to protocol (Fig. 1). Ptch1 and wild-type littermate mice were euthanized under deep anesthesia with isoflurane. 2–11 wild-type mice and 6–19 Ptch1 mice were analyzed at each time point from at least two litters.

2.3. Genotyping

Animals were genotyped by PCR amplification of genomic DNA extracted from the tail. The wild type allele was distinguished with primers 5′-CTG CGG CAA GTT TTT GGT TG-3′ and 5′-AGG GCT TCT CGT TGG CTA CAA G-3′, which yield a 200-bp PCR product. The mutant allele was detected using primers 5′-GCC CTG AAT GAA CTG CAG GAC G-3′ and 5′-CAC GGG TAG CCA ACG CTA TGT C-3′, which yield a 479-bp PCR product.

2.4. BrdU labeling

To examine migration of GCPs, a single intraperitoneal injection of 100 mg/kg body weight of 5-Bromo-2′-deoxyuridine (BrdU, CAS No. 59-14-3 Sigma–Aldrich, MO, USA) in saline (Otsuka Pharmaceutical Factory, Inc., Japan) was given to mice at PND8 and 14. Animals treated with BrdU at PND8 were euthanized as above 1.5 h after the injection and at PND10, 12, 14, 16, 18, and 21. Animals treated with BrdU at PND14 were euthanized 1.5 h after injection and at PND16 and 21 (Fig. 1).

Table 1
Primary antibodies used for immunohistochemistry.

Antigen	Clone	Concentration/dilution	Antigen retrieval	Visualization system	Source
BrdU	BU1/75(ICR1)	1 μg/mL	Autoclave	LSAB	AbD serotec
Ki-67	TEC-3	30 μg/mL	Autoclave	LSAB	Dako
NeuN	A60	3 μg/mL	Autoclave	Polymer	Millipore
$p27^{kip1}$	EP233(2)Y	1:2000	Autoclave	Polymer	Abcam
Nestin	Rat-401	3 μg/mL	Autoclave	Polymer	Millipore
CyclinD1	EPR2241(IHC)-32	1:300	Autoclave	Polymer	Millipore
GFAP	Polyclonal	1 μg/mL	Microwave	Polymer	Dako
Calbindin-D-28K	CB-955	3 μg/mL	Microwave	Polymer	Sigma–Aldrich

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