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## Effect of Estrogen on Ontogenic Expression of Progesterone and Estrogen Receptors in Rat Uterus

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**ABSTRACT**—The ontogenic expression of progesterone and estrogen receptors (PR and ER) and effect of estrogen on these receptors were investigated immunohistochemically in rat uterus from the day of birth (=0 day) to 30 days of age. Uterine epithelial and stromal cells showed a negative PR immunoreaction at 0 day. The PR in the epithelial cell nuclei appeared by 5 days, while the stromal cells showed a negative PR reaction until 12 days. The staining of the stromal cells appeared from 12 to 15 days. In both the epithelial and stromal cells, the initiation of the PR appearance was not affected by ovariectomy performed at 0 day or 5 days prior to the appearance of PR in the epithelial and stromal cells. Estrogen injections from 0 day failed to initiate the appearance of PR in the epithelial cells, regardless of doses of estradiol-17 $\beta$  (0.1, 1 and 10  $\mu$ g daily), but induced PR in the stromal cells. The staining of ER appeared at 5 days in the epithelial cells and at 1 day in the stromal cells, respectively. ER appeared after 2–3 daily injections of estrogen from 0 day depending upon the doses. These results suggest that steroid hormones secreted from neonatal ovary do not play any important role in ontogenic expression of PR during the postnatal uterine maturation.

### INTRODUCTION

It is well known that the rodent uterus is differentiated from the Müllerian duct during the late pregnancy and is developed sequentially after birth. In rats, differentiation of glandular epithelium and musculature occurs postnatally (Bigsby and Cunha, 1985; Branham *et al.*, 1985a,b), and the ability in uterine response to exogenous estrogen is incomplete in the neonates (Luck *et al.*, 1973; Sömjen *et al.*, 1973; Katzenellenbogen and Greger, 1974; Lyttle *et al.*, 1979; Sheehan *et al.*, 1981).

In mice, the uterus grows independently of estrogen in early postnatal life (Bigsby and Cunha, 1985). Li (1994) suggests that the mouse uterine epithelium proliferates in the absence of steroid hormones at least by two weeks after birth. By contrast, Sananès and Le Goascogne (1976) have demonstrated that a period from 7 through 10 days of age is particularly significant in the postnatal rat uterine development, during which endogenous estrogen plays a key role (Sananès *et al.*, 1980). Since estrogen action is mediated by an estrogen receptor (ER) in target cells, ontogenic changes in ER may be involved in the postnatal development of the uterus. In mice, ER is detected by autoradiographic analysis in stromal cells of Müllerian ducts from the 13th day of pregnancy (Holderegger and Keefer, 1986), whereas in epithelial cells of the uterus, ER appears after 18 days of age (Cunha *et al.*, 1982). Immunohistochemical study reveals that uter-

ine epithelial cells do not produce ER until 4 (Yamashita *et al.*, 1989, 1990), 5 (Bigsby *et al.*, 1990; Sato *et al.*, 1992) or 13 days of age (Li, 1994). In rats, the neonatal uterus contained nuclear ERs (Sömjen *et al.*, 1973) and the maturation of ER is completed within the first 5 days after birth (Csaba, 1980; Csaba and Incze-Gonda, 1993).

In rats, moreover, a critical period of the uterine ability responding to progesterone has been found in a postnatal life (Ohta, 1981). The rat uterine progesterone receptor (PR) has been reported to be absent in newborns (Nguyen *et al.*, 1988) and markedly lower in infants than in adults (Milgrom and Baulieu, 1970). It seems likely that development of PR, as well as ER, is involved in a requirement for a postnatal factor to induce uterine maturation. Although immunohistochemical localization of PR is demonstrated in the uterus of neonatal rats (Ohta *et al.*, 1993b) and mice (Li, 1994), ontogeny of PR in rat uterus is not duly studied.

The present study was planned to examine the expressions of PR and ER immunohistochemically in rat uterus during the postnatal development. Since PR in the adult rat uterus is under estrogenic control (Castellano-Diaz *et al.*, 1987), effects of ovariectomy and estrogen injections on uterine PR expression during the postnatal development were also examined in this study.

### MATERIALS AND METHODS

#### Animals

Female rats of the T strain used in the present experiments were maintained in a temperature- and light-controlled room (22 $\pm$ 2°C,

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lights on from 05:00 to 19:00). A standard laboratory pellet diet (Clea Co., Tokyo, Japan) and water were available *ad libitum*. All procedures were carried out according to the NIH Guide for the Care and Use of Laboratory Animals.

In the first series of experiments, PR and ER expressions in the intact rat uterus were examined at 0 (=the day of birth), 1, 2, 3, 4, 5, 7, 10, 12, 15, 20, 25 and 30 days of age.

In the second series of experiments, PR expression was examined in rats ovariectomized at 0, 10 or 20 days of age. The rats ovariectomized at 0 day were sacrificed at 5, 7, 10, 15, 20 and 25 days. Those ovariectomized at 10 or 20 days were killed 5 days after the operation.

In the 3rd series of experiments, the females were given 1 to 5 daily subcutaneous injections of 0.1, 1 and 10  $\mu$ g estradiol-17 $\beta$  (E<sub>2</sub>, Sigma Chemical Co., St. Louis, MO, USA) dissolved in 0.05 ml sesame oil or of vehicle only from 0, 5 or 10 days onward. These rats were sacrificed on the day after the last injection, and the PR expression was examined. Five-day-old rats given 3 daily injections of 10  $\mu$ g E<sub>2</sub> from 0 day were also sacrificed to examine PR expression. In addition, ER expression was examined in the uteri of females receiving 5 daily injections of 3 different doses of E<sub>2</sub> or of oil from the day of birth.

More than 3 animals were used for each point of data. At autopsy, uterine tissues embedded in O.T.C. compound (Miles Laboratories, Elkhart, IN, USA) were frozen in liquid N<sub>2</sub> and stored at -80°C until use.

*Immunohistochemical staining of PR and ER*

Anti-PR and -ER monoclonal (mouse) antibodies were purchased from Affinity BioReagents Inc. (Cat. No. MA1-410, Neshanic Station, NJ, USA) and Dako Japan Co. Ltd. (Dako-ER, 1D5, Kyoto, Japan), respectively; these antibodies were diluted with phosphate-buffered saline (PBS) containing 1% bovine serum albumin fraction V (BSA, Sigma Chemical Co., St. Louis, MO, USA) to give final concentrations of 5  $\mu$ g IgG<sub>1</sub>/ml for anti-PR antibody and 14.5  $\mu$ g IgG<sub>1</sub>/ml for anti-ER antibody, respectively. The specificity of these antibodies has been described in detail (for anti-PR antibody see Traish and Wotiz, 1990; for anti-ER antibody see Saati *et al.*, 1993). Horseradish peroxidase (HRP)-labeled anti-mouse IgG goat F(ab')<sub>2</sub> [HRP-F(ab')<sub>2</sub>] was obtained from Amersham Co. (Chicago, IL, USA). HRP-F(ab')<sub>2</sub> was diluted 50-fold with 1% BSA in PBS.

Frozen sections cut at 10  $\mu$ m by a cryostat-microtome were fixed in Zamboni's solution (for PR) or 4 % paraformaldehyde in

0.1 M phosphate buffer at pH 7.4 (for ER) for 10 min at room temperature, and then washed with PBS. The sections were immunohistochemically stained with anti-PR or -ER antibody as previously described (Ohta *et al.*, 1993a). Sections were treated with anti-PR or -ER monoclonal antibody for 16 hr at 4°C and, after washing with PBS, were incubated with HRP-F(ab')<sub>2</sub> for 2 hr at room temperature. Immunoreaction of PR or ER was visualized with diaminobenzidine tetrahydrochloride by the method of Straus (1982). Control sections were incubated with normal mouse serum instead of anti-PR or -ER antibody. In 3 slides of the uterus from each animal, the staining intensity of PR and ER was estimated severally by three investigators, and the averaged data represent a consensus value of the observations. The data were improved by a measurement of optical density of the cells (20 to 50 cells in 3 different fields) by a Color Image Analyzer CIA (Olympus, Tokyo, Japan). The staining was graded as (-) for negative staining, (+) for weak-positive staining, (++) for moderate-positive staining and (+++) for intense-positive staining.

**RESULTS**

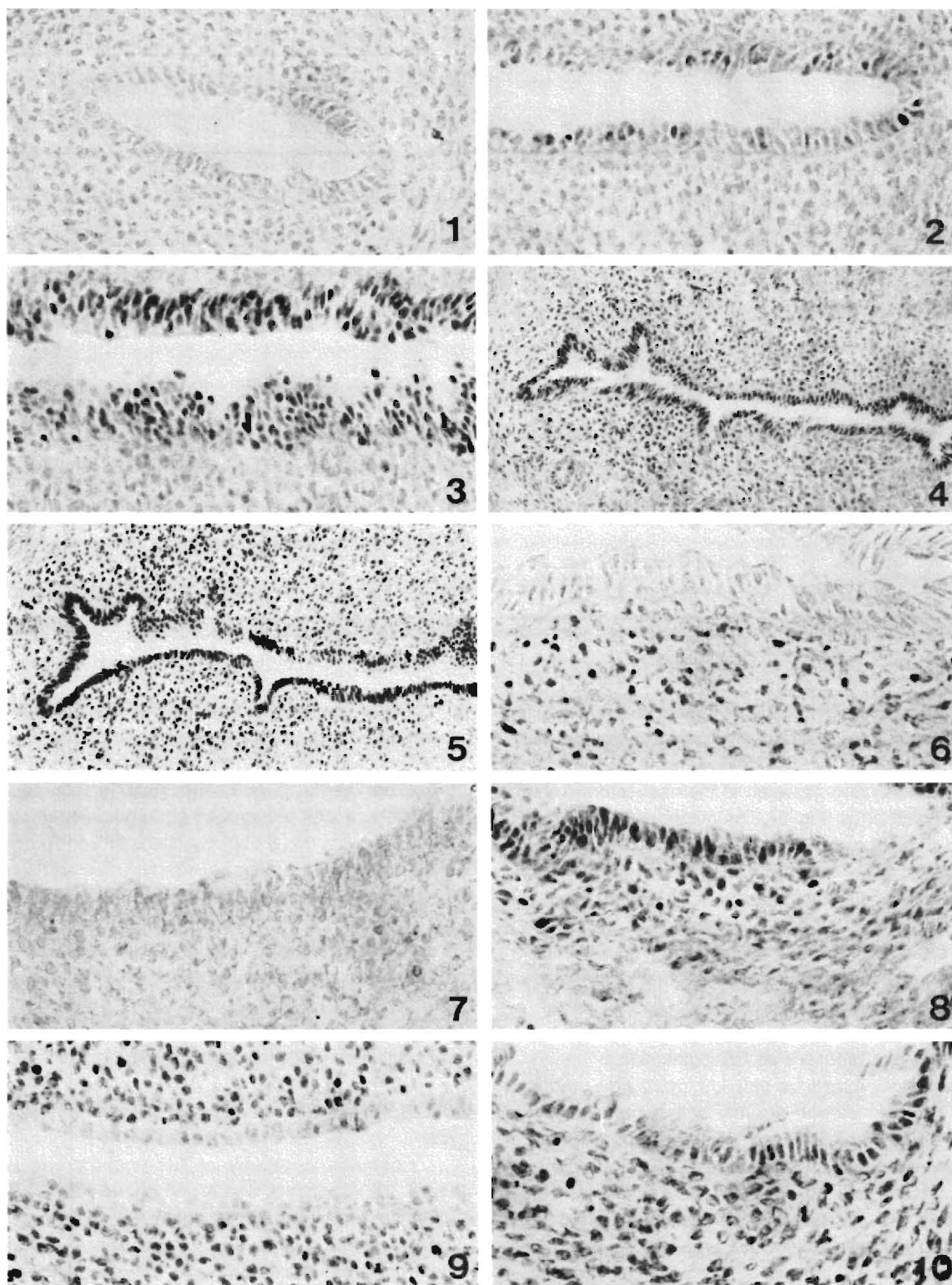
*Ontogenic expression of PR in the uterus of intact and ovariectomized rat*

The uterine stromal cells were not distinguishable from smooth muscle cells until the endometrial stroma and myometrium were differentiated from the mesenchyme after 5 days of age. Uterine epithelial and stromal (mesenchymal) cells showed a negative PR immunoreaction at 0 day. The epithelial cells exhibited no PR staining from 0 to 4 days, although at 5 days, a slight staining appeared in the nuclei (Table 1) (Figs. 1 and 2). The staining intensity gradually increased from 7 to 15 days (Figs. 3 and 4), and remained unchanged until 30 days. By contrast, the stromal cells showed a negative PR reaction until 12 days. Positive staining of the stromal cells was observed from 12 to 15 days, and then, increased steadily until 30 days. Muscle cells showed no definite positive staining until 20 days. The intensity of these cells was slightly increased by 30 days, but was slightly weaker than that in the epithelial and stromal cells.

Table 1. Postnatal ontogeny of progesterone receptor immunoreactivity in the uterus of the intact and ovariectomized rat

Cell type	Age in days												
	0	1	2	3	4	5	7	10	12	15	20	25	30
Intact													
Ep	-	-	-	-	-	-/+	+ /++	++	++	+++	+++	+++	+++
St	-	-	-	-	-	-	-	-	-/+	++	++	++	++
Ms	UD	UD	UD	UD	UD	-	-	-	-	-/+	+	++	++
Ovariectomy at 0 day of age													
Ep						-/+	+ /++	++		+++	+++	+++	
										(+++) <sup>a</sup>		(+++) <sup>b</sup>	
St						-	-	-		++	+++	+++	
										(++) <sup>a</sup>		(++) <sup>b</sup>	
Ms						-	-	-		-/+	+	+	
										(-/+) <sup>a</sup>		(+) <sup>b</sup>	

Ovariectomy was carried out at 10 (a) and 20 (b) days of age, respectively.  
Ep, epithelial cells; St, stromal cells; Ms, muscle cells; UD, undistinguishable from stromal cells.  
-, +, ++ and +++ indicate negative, moderate and intense staining, respectively.



- Fig. 1. Uterus from a 0-day-old rat with immunostaining of progesterone receptor (PR). Nuclear staining is undetectable.  $\times 300$ .
- Fig. 2. Uterus from a 5-day-old rat with immunostaining of PR. The staining is discernible only in the epithelial cell nuclei.  $\times 300$ .
- Fig. 3. Uterus from a 10-day-old rat with immunostaining of PR. The staining is detected only in the epithelial cells.  $\times 300$ .
- Fig. 4. Uterus from a 15-day-old rat with immunostaining of PR. Positive staining is observed in both the epithelial and stromal cells.  $\times 150$ .
- Fig. 5. Uterus from a 15-day-old rat ovariectomized at 0 day. Intense immunostaining of PR is discernible in both the epithelial and stromal cells (compare with Fig. 4).  $\times 150$ .
- Fig. 6. Uterus from a 3-day-old rat given 3 daily injections of  $10 \mu\text{g E}_2$  from 0 day. Positive PR immunostaining is observed in the stromal cells.  $\times 300$ .
- Fig. 7. Uterus from a 5-day-old rat given 3 daily injections of  $10 \mu\text{g E}_2$  from 0 day. Neither the epithelial nor the stromal cells show the PR staining.  $\times 300$ .
- Fig. 8. Uterus from a 10-day-old rat given 5 daily injections of  $0.1 \mu\text{g E}_2$  from 5 days. The PR staining is found in both the epithelial and stromal cells (compare with Fig. 3).  $\times 300$ .
- Fig. 9. Uterus from a 2-day-old rat with immunostaining of estrogen receptor (ER). The staining is discernible only in the stromal cells.  $\times 300$ .
- Fig. 10. Uterus from a 2-day-old rat given 2 daily injections of  $10 \mu\text{g E}_2$  from 0 day. The ER staining is detectable in both the epithelial and stromal cells (compare with Fig. 9).  $\times 300$ .

Table 2. Effects of 5 daily injections of estrogen starting at 0 (=day of birth), 5 and 10 days of age on progesterone receptor expression in rat uterus

Doses of E <sub>2</sub> (μg)	Cell type	Ages in days														
		E <sub>2</sub> or oil injection (0–4 days)					E <sub>2</sub> or oil injection (5–9 days)					E <sub>2</sub> or oil injection (10–14 days)				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
control																
oil	Ep	—	—	—	—	—/+	+	+/++	++	++	++	++	++	++	++	++
	St	—	—	—	—	—	—	—	—	—	—	—	—/+	+	+	++
	Ms	UD	UD	UD	UD	—	—	—	—	—	—	—	—	—	—	—/+
estrogen injections from 0, 5 or 10 days of age																
0.1	Ep	—	—	—	—	—	+	+/+++	+/+	+++	++	++	+	+	+	—
	St	—	—	+	+	+	+	+	++	++	++	++	++	++	++	++
	Ms	UD	UD	UD	UD	+	—	+	+	++	++	+	+	+	++	++
1	Ep	—	—	—	—	—	+	+/++	++	++	+	++	—	—	—	—
	St	—	—	+	+	+	+	++	++	++	++	++	++	++	++	++
	Ms	UD	UD	+	+	++	+	++	++	++	++	+	++	++	++	+++
10	Ep	—	—	—	—	—	+	—	—	—	—	++	—	—	—	—
	St	—	++	++	++	+	+	++	++	++	+	++	++	++	++	++
	Ms	UD	UD	++	++	++	+	+	++	++	++	++	++	+++	++++	++++

E<sub>2</sub>, estradiol-17β; Ep, epithelial cells; St, stromal cells; Ms, muscle cells; UD, undistinguishable from stromal cells.

In rats ovariectomized at 0 day, uterine cells showed the PR expression pattern similar to those in the intact rats. After 15 days, the intensity of PR staining in the epithelial and stromal cells was rather stronger in the ovariectomized rats than in the intact ones (Fig. 5). The mesenchyme, however, developed into the stroma and the myometrium in these ovariectomized rats during the same postnatal period as seen in the intact rats. Ovariectomy at 10 and 20 days also failed to exert any effects on PR expression appearing 5 days after the operation in the epithelial and stromal cells. In the muscle cells, the staining was slightly decreased by ovariectomy at 20 days compared to that in intact uterus.

#### Effects of estrogen injections on PR expression

Two or 3 daily injections of 0.1, 1 or 10 μg E<sub>2</sub> induced PR staining in the uterine stromal cells when they were given from 0 day (Table 2; Fig. 6). When commencement of the estrogen injection was postponed to 5 or 10 days, PR staining appeared in the stromal cells after a single injection regardless of the doses of E<sub>2</sub>. The staining intensity of stromal cells responding to 0.1 or 1 μg E<sub>2</sub> during 0 to 5 days was lower than that during 5 to 15 days. The intensity of stromal cells decreased after 5 daily injections of 10 μg E<sub>2</sub> starting at 0 day when compared to that after 4 daily injections. In any dose, however, the estrogen injections from 10 days showed no increase in the staining intensity of the stromal cells compared to the cells in 15-day-old intact rats. The staining of PR in the stromal cells induced by the 3-day treatment with 10 μg E<sub>2</sub> from 0 day disappeared by 5 days (not shown in Table 2) (Fig. 7).

The myometrial differentiation was initiated by 3 daily injections of 1 or 10 μg E<sub>2</sub> starting at 0 day. The estrogen injection produced PR in the muscle cells throughout almost the experimental period. The staining was elevated with increasing doses of estrogen and age; intense staining was found following 3 to 5 daily injections of 1 or 10 μg E<sub>2</sub> starting at 10 days.

By contrast, the PR staining in the epithelial cells was not induced by any of the estrogen treatment commencing at 0 day. The PR staining in the epithelial cells was inhibited by all estrogen treatments at 10 or 15 days, whereas treatments with 0.1 and 1 μg E<sub>2</sub> beginning at 5 days had little effects on PR staining normally appearing in the epithelial cells between 6 and 10 days (Fig. 8).

#### ER expressions in the uterus of intact and estrogen-treated rats

Postnatal ontogeny of ER immunoreactivity in the uterus of intact rats was summarized in Table 3. Uterine epithelial and stromal (mesenchymal) cells showed no ER immunostaining at 0 day. In the epithelial cells, nuclei showed a slight staining at 5 days and then gradually increased in stainability with age. By contrast, the stromal cells showed a positive staining from 1 day (Fig. 9); the staining intensity was almost unchanged until 15 days and thereafter slightly increased. The muscle cells showed a similar ER expression in the postnatal development to the stromal cells. In rats given daily injections of 1 or 10 μg E<sub>2</sub> from 0 day, ER appeared in the epithelial cells at 2 days (Fig. 10), while in those given 0.1 μg E<sub>2</sub>, 3 days were needed

Table 3. Postnatal ontogeny of estrogen receptor immunoreactivity in the uterus of the intact rat

Cell type	Age in days												
	0	1	2	3	4	5	7	10	12	15	20	25	30
Ep	—	—	—	—	—	—/+	+/++	++	++	++	++	++	+++
St	—	+/++	++	++	++	++	++	+	++	++	++	++	++
Ms	UD	UD	UD	UD	UD	++	++	+	++	++	++	++	++

Ep, epithelial cells; St, stromal cells; Ms, muscle cells; UD, undistinguishable from stromal cells.

Table 4. Effects of 5 daily injections of estrogen beginning at 0 day on estrogen receptor expression in rat uterus

Doses of E <sub>2</sub> (μg)	Cell type	Age in days				
		1	2	3	4	5
oil	Ep	—	—	—	—	—/+
	St	+	+	+/++	++	++
	Ms	UD	UD	UD	UD	+/++
0.1	Ep	—	—	+	+	+
	St	+	+	+/++	++	++
	Ms	UD	UD	UD	UD	+
1	Ep	—	+	+	+	+
	St	+	+	+/++	++	++
	Ms	UD	UD	+	+	+
10	EP	—	+	+	+	+
	St	+	+	+/++	+/++	+/++
	Ms	UD	UD	+	+	+

E<sub>2</sub>, estradiol-17β; Ep, epithelial cells; St, stromal cells; Ms, muscle cells; UD, undistinguishable from stromal cells.

to induce ER in the epithelial cells (Table 4). The staining intensity was approximately the same between groups, regardless of doses of estrogen. The neonatal injections of estrogen had little effects on the ER staining in the stromal and muscle cells.

## DISCUSSION

In rat uterus, ER is known to increase in number after birth and reach a maximum by 10 days of age (Clark and Gorski, 1970), while PR is not detectable in newborns (Nguyen *et al.*, 1988) and the progesterone-binding sites are markedly lower in infants than in adults (Milgrom and Baulieu, 1970). These findings indicate that the ability of endometrial cells to synthesize receptor proteins may be involved in the uterine maturation. In the present study, immunohistochemical methods employing antibodies to PR and ER have been used to study the ontogeny of these receptors in the rat uterus. A recent immunohistochemical study demonstrated that in the mouse uterus, PR occurred from the day of birth in both epithelial and stromal cells (Li, 1994). In the present study, however, the rat uterus was devoid of PR on the day of birth as reported by Nguyen *et al.* (1988) and Ohta *et al.* (1993b). In the epithelial cells, the PR staining appeared by 5 days, while in the stromal cells, the staining was not observed until 12 days. In addition to the differentiation of musculature, the postnatal ontogeny of PR expression in both the epithelial and stromal cells was not

influenced by ovariectomy at 0 day or 5 days prior to the appearance of PR. This is in good agreement with the findings that the uterine growth as estimated by the amount of DNA, protein content and weight gain is not affected by ovariectomy until 10 to 20 days after birth (Clark and Gorski, 1970; Döcke *et al.*, 1981), and that ovariectomy at 0 day does not interfere with decidualoma formation in adult rats given injections of progesterone and estrogen (Ohta, 1982).

The present findings indicate that estrogen injection from the day of birth enhanced musculature differentiation expressing both ER and PR, however, it could not produce PR and rather suppress the appearance in the epithelial cells, when given in later period. The ER in the epithelial cells was absent for the neonatal period and was induced by estrogen injections from 0 day. It is notable that in rats, the neonatal injection of estrogen failed to initiate PR production in the epithelial cells despite success of the ER appearance in these cells by the injection. The difference between the ER and PR induction by estrogen in the epithelial cells, therefore, may be ascribable to the difference in the mechanism involving the receptor induction by estrogen on these cells. In rats ovariectomized as adults, estrogen injections decreased the PR in the epithelial cells but increased it in the stromal cells (Ohta *et al.*, 1993a).

Cunha *et al.* (1983) showed that stromal cells induced epithelial cell growth and differentiation in various reproductive organs. Since ER was present only in uterine

stromal cells at the day of birth, it is conjectured that estrogen acts directly on the stromal cells, resulting in the induction of ER expression of epithelial and muscle cells. By contrast, "matured" uterine stromal and muscle cells stimulated by high doses of estrogen suppress induction of PR in epithelial cells and/or such estrogen directly inhibits PR production in epithelial cells. Sato *et al.* (1992) suggested that in mice, the expression of ER in uterine epithelial cells is mediated by EGF inasmuch as the effect of estrogen on proliferation of uterine epithelial cells is reported to be mediated by epidermal growth factor (EGF) *in vivo* and *in vitro* (Tomooka *et al.*, 1986; DiAugustine *et al.*, 1988; Uchima *et al.*, 1991). At present, however, an involvement of EGF in the PR expression is unclear in rat uterus, although 3 daily injections of 10 µg mouse EGF from 0 day failed to induce PR in any of rat uterine cells (Ohta, unpublished data). In this connection, experiments with EGF are now under way.

Estrogen injections of low doses during the period of 5 to 9 days after birth, unlike the other injection period, induced the PR staining in the stromal cells without affecting expression of the PR staining normally appearing in the epithelial cells during this period, indicating that the stromal cells change to the "matured" stromal cells during this period. This is consistent with the findings that the postnatal period of 5 or 7 to 10 days has particular significance for the maturation of the rat uterus (Ohta, 1981; Sananès and Le Goascogne, 1976). The low responsibility of stromal cells to estrogen producing PR until 5 days may account, at least in part, for the failure of decidual response to uterine trauma during this period in rats given progesterone and estrogen (Ohta, 1981). It is concluded, therefore, that the ability of endometrial cells to synthesize PR is involved in the uterine maturation. On the other hand, Kennedy (1985) suggests that prostaglandins play a role in the induction of decidual reaction. It may be of interest to study changes in uterine prostaglandin synthesizing ability during the postnatal development.

The estrogen injection produced the PR staining in the stromal cells regardless of ages at commencement of the treatment. In any dose, however, the estrogen injections failed to cause the PR level over the values after 12 days in the stromal cells. The PR in the stromal cells induced by the 3 daily injections from 0 day disappeared at 5 days. Although progesterone is reported to increase the PR staining of the epithelial cells in rats ovariectomized as adults (Ohta *et al.*, 1993a), neonatal injections of 10 µg progesterone could not induce PR in the epithelial and stromal cells of rats (Ohta, unpublished data).

The present findings indicate that in rats, ovarian steroids secreted from neonatal ovary do not play any important role in ontogenic expression of PR during the postnatal uterine maturation. It is probable, therefore, that the PR develops autonomously in the postnatal uterine cells as have been suggested in the ER of developing rat uterus by Clark and Gorski (1970).

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