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Original

α-Smooth Muscle Actin-positive Stromal Cells Reactive to Estrogens Surround Endometrial Glands in Rats but not Mice

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Abstract: In human endometrium, α-smooth muscle actin (α-SMA)-positive stromal cells (SMA-SCs) surround endometrial glands, and the α-SMA expression is regulated by estrogen. The biological significance of these cells remains to be elucidated, and no information is available with regard to their animal counterparts. The present study, therefore, investigated SMA-SCs in the uteri of female Donryu rats and CD-1 mice. SMA-SCs with morphological similarities to those in the human were detected around the endometrial glands in normal cycling rats, but not in mice. Furthermore, the rat SMA-SCs disappeared after ovariectomy but returned with estrogen replacement in a duration-dependent manner, suggesting the regulatory role of estrogens similar to the human situation. Thus, SMA-SCs are present in rats, but not in mice, with characteristics close to their human counterparts. Their biological significance now needs to be elucidated by comparative studies. (J Toxicol Pathol 2005; **18**: 47–52)

Key words: endometrial stromal cells, α-smooth muscle actin, 17β-estradiol, octylphenol, Donryu rat

Introduction

Actin, a cytoskeletal protein involved in cell contraction, cell movement and cell-to-substrate adhesion[1–6], has been divided into six isoforms: two non-muscle actins (β and γ) known to be cytoplasmic, two smooth muscle actins (α and γ), and two sarcomeric actins (α-cardiac and α-skeletal)[7–9]. The α-smooth muscle actin (α-SMA) is found in smooth muscle cells, pericytes and myoepithelial cells[10–12], and in humans normal endometrial stromal cells around endometrial glands have also been shown to immunostain for α-SMA[13,14]. This α-SMA expression in stromal cells changes during the estrous cycle, greater numbers of positive cells being present in the proliferative than in the secretory phase[13]. Furthermore, in the proliferative phase α-SMA-positive stromal cells (SMA-SCs) can be detected occasionally in the more superficial mucosa and around non-dilated glands as well as in the lower, basal layer of the endometrial mucosa and around dilated or cystic glands, whereas SMA-SCs in the secretory phase are mostly evident in the basal, inactive layer and around single non-secretory glands[14]. This suggests that estrogen influences α-SMA expression in the endometrial stromal cells, although their significance remains to be elucidated. Furthermore, no information is available in the literature about their existence in experimental animals, such as rodents. The present study was thus conducted to determine whether SMA-SCs are a feature of the uteri of rats and mice. Finding them present in rats, we then assessed the reactivity of SMA-SCs to 17β-estradiol and *p-tert*-octylphenol, an endocrine disrupting chemical with estrogenic activity, using ovariectomized rats.

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Materials and Methods

Ethical considerations for animal experiments

The animal experiments conducted in this study were approved by the Animal Experimentation Committee of the Sasaki Institute prior to their execution and were conducted under monitoring by the committee in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, Japanese Government Animal Protection and Management Law Number 105 and Japanese

Government Notification on Feeding and Safekeeping of Animals Number 6.

Animals

A total of 30 virgin female Donryu rats (Crj:Donryu, 6 or 10 weeks of age) and 12 virgin CD-1 mice (Crj:CD-1, 7 weeks of age) were purchased from Charles River Japan Inc. (Kanagawa, Japan). They were housed in plastic cages, kept in an air-conditioned animal room (under constant conditions of 24 ± 2°C, 55 ± 10% humidity, and a 12-hour light/dark cycle), and maintained on a basal diet, CRF-1 (Oriental Yeast Inc., Tokyo, Japan) with tap water *ad libitum*.

Experimental design

Experiment I: Twelve animals each of the two species showing normal estrous cyclicity were selected, and vaginal smears were checked every morning. At ages of 12 or 15 weeks (rats) and 9 weeks (mice), 3 animals each were euthanized in the 4 stages of the estrous cycle (proestrus, estrus, metestrus and diestrus), and the uteri were excised for histological and immunohistochemical examination. The uterine horns were fixed in 10% neutrally buffered formalin solution and embedded in paraffin. Appropriate numbers of serial sections at a thickness of 4 μm were then prepared from each specimen, one being routinely stained with hematoxylin and eosin for histological examination. The other sections were processed for immunohistochemical analyses using mouse monoclonal antibodies against α-SMA (clone 1A4, Dakocytomation Japan, Kyoto, Japan, 100-fold diluted) and cytokeratin 14 (CK14; clone LL002, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK, 20-fold diluted), the latter for the rat uteri only, at 4°C overnight, then processed for the labeled polymer method using an Envision Plus kit (Dakocytomation) according to the manufacturer's instructions, and counterstained with hematoxylin. Negative controls were included with primary antibodies omitted.

Experiment II: At 8 weeks of age, 18 rats underwent ovariectomy via the dorsal route under light ether anesthesia. Three weeks after the operation, they were equally divided into 3 groups, given subcutaneous injections of vehicle (dimethylsulfoxide), 5 μg/kg/day of 17β-estradiol (E_2; Wako Pure Chemical Industries Ltd., Osaka, Japan) or 100 mg/kg/day of *p-tert*-octylphenol (OP; Wako), respectively, for 2 or 14 successive days (3 animals each). The animals were euthanized 24 hours after the last administration, and the uteri were excised. The uterine horns were processed in the same manner as for experiment I except that CK14 immunostaining was not performed.

Results

Experiment I

In rats, the endometrial stroma was cellular, especially in the subluminal layer surrounding the glandular epithelium. The stromal cells surrounding the glands were spindly in shape with oval or spindle nuclei, resembling fibroblasts rather than basket-shaped myoepithelial cells. They were arranged in one or several layers around the glands (Fig. 1A) in all stages of the estrous cycle. In mice, the endometrial stroma was also cellular, but stromal cells surrounding the endometrial glands were not very conspicuous (Fig. 2A).

Immunohistochemically, the stromal cells surrounding the endometrial glands in one or several layers were positive for α-SMA in rats (Figs. 1B and 1C). Positive cells were observed through all stages of the estrous cycle, but the cell layers were thickest in proestrus (Figs. 1B and 1C). The stromal cells were negative for CK14 (Fig. 1D). In mice, SMA-SCs were not detected at any stage of the estrous cycle (Fig. 2B). In the uteri of both rats and mice, smooth muscle fibers in the blood vessels and the myometrium were positive for α-SMA (Figs. 1B, 1C and 2B).

Experiment II

In the ovariectomized rats, the uteri were severely atrophic, and the luminal epithelial cells in the endometrium were cuboidal rather than columnar in shape. The endometrial stromal cells and smooth muscle in the myometrium were reduced in size (Fig. 3A). In rats treated with E_2 for 2 days, the size of the uteri recovered remarkably, and the luminal epithelial cells in the endometrium were again columnar in shape. The endometrial stromal cells also recovered, and the myometrium was multi-layered (Figs. 3A and 3B). In rats treated with E_2 for 14 days, the uteri were as large as those of 2-day-treated animals while the endometrial stromal cells were larger (Figs. 3B and 3C) and the myometrium was thicker (Figs. 3B and 3C). In rats receiving OP, the uteri generally demonstrated similar histological findings to those in the rats treated with E_2 for the same term (Figs. 3C and 3D).

Table 1 summarizes the data of the α-SMA immunohistochemistry of stromal cells surrounding the endometrial glands in experiment II. In ovariectomized rats treated with vehicle stromal cells were negative for α-SMA (Fig. 4A), but 2 out of 3 rats treated with E_2 for 2 days exhibited weakly-positive spindle cells around the glands (Fig. 4B). Furthermore, α-SMA-positive cells were observed in all rats injected with E_2 for 14 days (Fig. 4C). Similarly, one out of 3 rats treated with OP for 2 days and all rats receiving OP for 14 days had SMA-SCs (Fig. 4D). Smooth muscle fibers in the blood vessels and the myometrium were positive for α-SMA in rats of all groups (Figs. 4A-D).

Discussion

The present study unequivocally demonstrated the presence of SMA-SCs surrounding endometrial glands in untreated rats but not mice. The SMA-SCs clearly differed from myoepithelial cells, characterized as basket-shaped with a positive CK14 phenotype[15,16], and were similar to SMA-SCs in the human endometrium, negative for

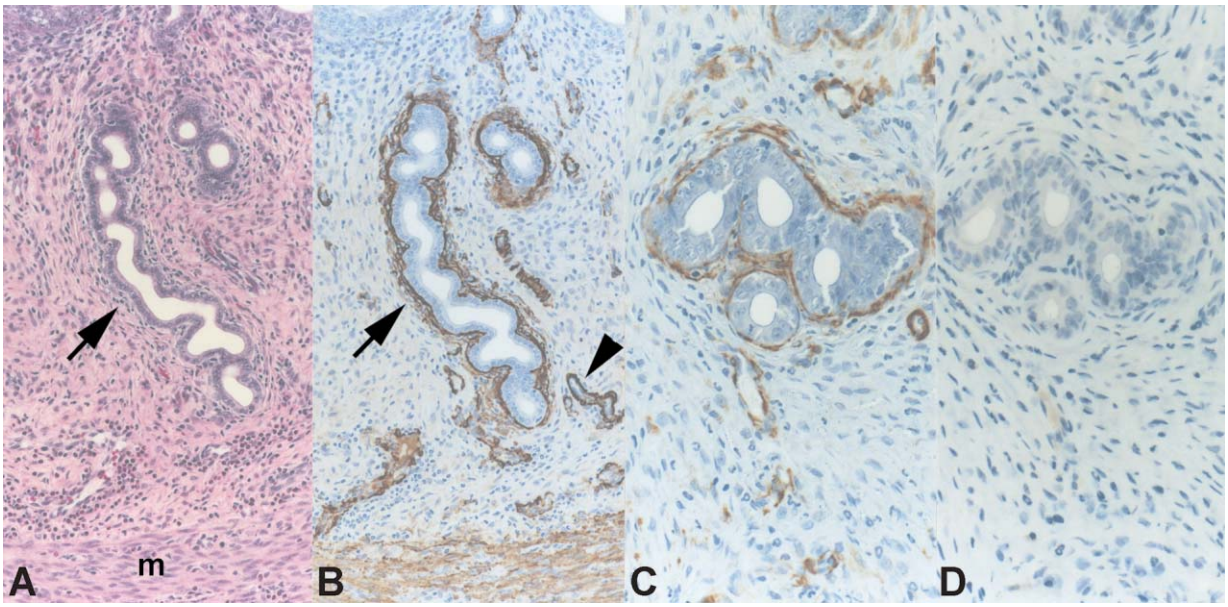

Fig. 1. Uteri of untreated rats (experiment I). (A) Representative histology in proestrus. Several layers of stromal cells surround endometrial glands (arrow). m: myometrium, × 90. (B) Representative α-SMA immunohistochemistry in proestrus. One or several layers of stromal cells surrounding endometrial glands are positive (arrow). Smooth muscle fibers in the blood vessels (arrowhead), as well as in the myometrium, are also positive, × 90. (C) Representative α-SMA immunohistochemistry in metestrus. One or two layers of stromal cells surrounding endometrial glands are positive, × 180. (D) Representative CK14 immunohistochemistry in metestrus. The endometrial stromal cells are negative, × 180.

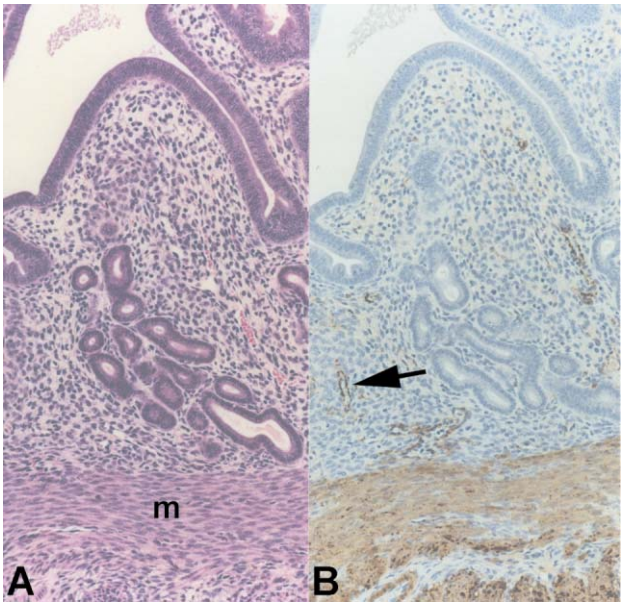

Fig. 2. Uteri of untreated mice (experiment I). (A) Representative histology in proestrus. Endometrial stromal cells are diffusely present throughout the stroma. m: myometrium, × 90. (B) Representative α-SMA immunohistochemistry in proestrus. The endometrial stromal cells are negative. Smooth muscle fibers in the blood vessels (arrow), as well as in the myometrium, are positive, × 90.

Table 1. Grades of α-SMA Immunohistochemistry for the Stromal Cells Surrounding Endometrial Glands in Ovariectomized Rats (Experiment II)

Group	Treatment		Grade		
	Compound	Period	–	±	+
1	Vehicle	2 days	3	0	0
		14 days	3	0	0
2	E_2	2 days	1	2	0
		14 days	0	0	3
3	OP	2 days	2	1	0
		14 days	0	0	3

Symbols used are: –, negative; ±, weakly positive; +, positive.

cytokeratins and distinguishable from myoepithelial cells[14]. Human SMA-SCs have been suggested to be a subset of myofibroblasts[13,14]. Myofibroblasts are characterized as having features of both smooth muscle cells and fibroblasts[17–19], and can be classified into 4 subtypes based on their differential immunoreactivity with antibodies against vimentin, desmin and α-SMA[20]. Results of the present study indicate that the SMA-SCs present in rats similarly have a myofibroblast origin, judging from the morphological findings.

In the rats, layers of SMA-SCs were apparent in proestrus, when serum E_2 levels are the highest of the estrous cycle. The fact that the endometrial stromal cells surrounding endometrial glands were small in size and negative for α-SMA in ovariectomized rats, with recovery

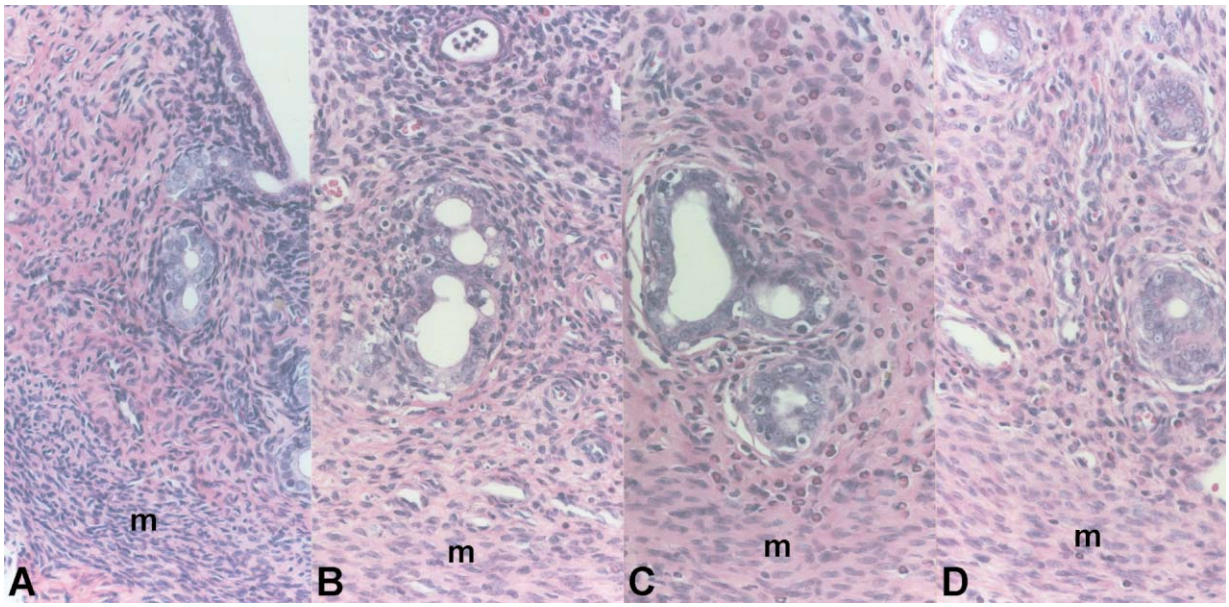

Fig. 3. Representative histology of uteri of ovariectomized rats (experiment II), × 180. (A) Treated with vehicle for 14 days. The endometrial stromal cells and smooth muscle in the myometrium (m) are small in size. (B) After treatment with E_2 for 2 days; the endometrial stromal cells are larger in size than those of the vehicle controls. Smooth muscle in the myometrium (m) is also thicker. (C) After treatment with E_2 for 14 days; the endometrial stromal cells are larger than those of the 2-day-treated rats and the myometrium (m) is thicker. (D) After treated with OP for 14 days; the endometrial stromal cells are large and the myometrium (m) is thick, like those of the rats receiving E_2 for 14 days.

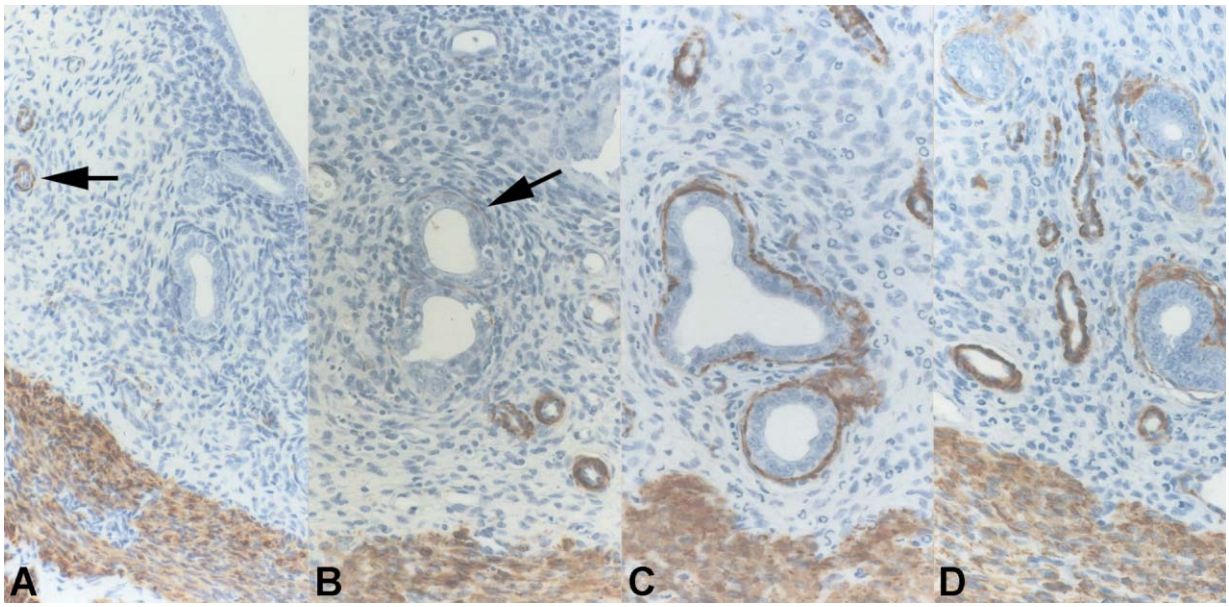

Fig. 4. Representative α-SMA immunohistochemistry of uteri of ovariectomized rats (experiment II), × 180. (A) Treated with vehicle for 14 days. Stromal cells surrounding endometrial glands are negative. Smooth muscle fibers in the blood vessels (arrow), as well as in the myometrium, are positive. (B) Treated with E_2 for 2 days. Some stromal cells surrounding endometrial glands are weakly positive (arrow). (C) Treated with E_2 for 14 days. One layer of the stromal cells surrounding endometrial glands is positive. (D) Treated with OP for 14 days. The stromal cells surrounding endometrial glands are positive.

on treatment with estrogen or OP, in a duration-dependent manner, clearly points to hormone dependence. OP is an endocrine disrupting chemical with estrogenic activity, from *in vitro* and *in vivo* evidence[21–28], and the dose of OP used in this study has been shown to be sufficient to exert estrogenic effects on the female reproductive tract in ovariectomized rats[27]. The results thus suggest that, similar to the human situation[13,14], estrogen modulates α-SMA expression in the

stromal cells surrounding endometrial glands in rats. Although the underlying mechanisms remain largely obscure, estrogen receptors can be immunohistochemically detected in endometrial stroma as well as in epithelium and in myometrium[29], and Hsu and Frankel[30] have demonstrated that mRNA expression of the *smooth muscle actin* gene is up-regulated by estrogens in immature rat uteri.

Myofibroblasts have been proposed as playing crucial roles in the contraction and relaxation of human granulation tissue on the basis of *in vitro* pharmacological reactivity similar to the smooth muscle[2]. α-SMA-positive myofibroblasts are also observed in rat granulation tissue[18] and may act similarly to their human counterparts. α-SMA is also expressed in passaged cultures of chick embryo fibroblasts[10], and stress fibers containing actin have been postulated as playing structural roles in the connection of the cytoplasmic matrix to the substrate rather than being contractile[4]. Thus, SMA-SCs surrounding endometrial glands might either participate in the contraction of glands or in the cell's adhesion to the surrounding substrate. Mechanistic studies are now needed to clarify, for example, the lack of SMA-SCs in mice.

In conclusion, SMA-SCs are present in rats, but not mice, and surround the endometrial glands, exhibiting morphological and endocrinological similarities to their human counterparts. Elucidation of their functional significance now needs to be performed by comparative studies in different species.

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