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INHIBITORY EFFECT OF ANDROGEN ON CELL DEATH OF MOUSE UTERINE EPITHELIUM

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Summary—The protective effect of androgen against the cell death of mouse uterine epithelium was evaluated by examining the retention of 5′-[^{125}I]iodo-2′-deoxyuridine ([^{125}I]IdUrd) incorporated into the whole uterus and the apoptotic index (percentage of the apoptotic cells to the total cells) which is a good index of physiological cell death. Castrated adult female mice were daily injected with oestradiol-17β for 3 days, followed by the injection of [^{125}I]IdUrd. Thereafter, these mice were daily injected with only the vehicle or 5α-dihydrotestosterone (DHT), and the ^{125}I-radioactivity retained in the whole uterus was determined. When only the vehicle was injected, the ^{125}I-radioactivity retained in the whole uterus rapidly decreased but injections of DHT reduced the loss of ^{125}I-radioactivity. The effect of DHT on the retention of ^{125}I-radioactivity depended on doses of DHT and was abolished by the pure antiandrogen, flutamide. The apoptotic index of uterine cells was examined by a similar experimental protocol, but without an injection of [^{125}I]IdUrd. Injections of only the vehicle caused marked increases in the apoptotic indices of both luminal and glandular epithelia, but injections of DHT decreased them significantly. The apoptotic index of stroma was not affected by the injection of DHT. The present results indicate that androgen reduces the cell death of mouse uterine epithelium through the androgen receptor.

INTRODUCTION

Atrophy of the uterus or the prostate after the castration of mature animals involves cell loss as well as diminution in cell size in these organs [1–6]. This suggests that steroids secreted from the ovary or testis play an important role in the maintenance of cells in their target organs. In fact, several investigations have shown that oestrogen or progesterone prevents the cell loss of the uterine epithelium [2, 4, 7–9] and that androgen decreases the cell loss of the seminal vesicles and prostate [1, 3, 5, 10, 11]. Thus, the prevention of cell loss is one of the important actions of steroids.

Cell death after the depletion of steroids in their target organs occurs as apoptosis, a scattered cell death [1–10]. Accordingly, the percentage of the apoptotic cells to the total cells has been used for the quantitative evaluation of the degree of the cell death [4, 6, 7, 9, 10]. On the other hand, as we have shown [9–11], the cell death in mouse organs can be easily and sensitively examined by measuring the retention of 5′-[^{125}I]iodo-2′-deoxyuridine ([^{125}I]IdUrd) incorporated into the whole organ.

Androgen plays little role in the control of the uterine growth of normal animals but exogenously administered androgen stimulates the uterine growth [12]. Although androgen maintains epithelial cells in the seminal vesicles and prostate [1, 3, 5, 10], the preventive effect of androgen on the cell death of the uterus remains unknown. Thus, we investigated the effect of androgen on the death of mouse uterine cells using both the morphological method and the method with [^{125}I]IdUrd.

EXPERIMENTAL

Chemicals

The chemicals were obtained from the following sources: [methyl-^{3}H]thymidine (86.4 Ci/mmol) from New England Nuclear Corp. (Boston, Mass, U.S.A.); 5′-[^{125}I]iodo-2′-deoxyuridine ([^{125}I]IdUrd; 2200 Ci/mmol) from Amersham International (Bucks, England); fluorodeoxyuridine, oestradiol-17β,5α-dihydrotestosterone (DHT), Polysorbate 80 and carboxymethylcellulose from Sigma Chemical Co. (St Louis, Mo., U.S.A.); and Sakura NR-M2 emulsion from Konica Industries (Tokyo, Japan). The pure antiandrogen, flutamide (α,α,α-trifluoro-2-methyl-4′-nitro-*m*-propionotoluidine, SCH 13521) was kindly supplied by Nippon Kayaku Co. Ltd (Chiyoda, Tokyo, Japan). All other reagents were of analytical grade.

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Mice

Female C57BL/6 mice at ages of 50–60 days were obtained from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). All the mice were castrated at the age of 60 days using pentobarbital sodium anaesthesia and used for experiments 28 days after the castration. Mice were kept at 25°C under a controlled light condition (12 h light/12 h darkness) and allowed free access to water and pellet food.

Injection of steroid and flutamide

Oestradiol-17β (1 μg), progesterone (1 mg), DHT (20–200 μg), and flutamide (2 mg) were suspended in 0.1 ml vehicle (0.9% NaCl, 0.4% Polysorbate 80, 0.5% carboxymethylcellulose, and 0.9% benzyl alcohol) and injected into the mice s.c.

Effect of DHT on the retention of [^{125}I]IdUrd incorporated into the whole uterus

Castrated female mice were daily injected with oestradiol-17β (1 μg/mouse/day) at 6 p.m. for 3 days, and at 8 a.m. on the day following the last injection of oestradiol-17β (day 1), all the mice were given an i.p. injection of 8 nmol/g body wt fluorodeoxyuridine to inhibit the endogenous thymidine synthesis [13]. After 1 h, 0.12 μCi/g body wt [^{125}I]IdUrd was injected i.p. Eight mice were killed 4 h after the [^{125}I]IdUrd injection, and the ^{125}I-radioactivity incorporated into the whole uterus was determined. The remaining mice were divided into 2 groups. The mice of each group were injected daily with only the vehicle or DHT (100 μg/mouse/day) at 6 p.m. from day 1. Eight mice of each group were killed following 3, 6, 9, 12 and 15 injections (on days 4, 7, 10, 13 and 16), and the ^{125}I-radioactivity retained in the uterus was determined. The ^{125}I-radioactivity retained in the whole uterus was determined as described previously [10, 11]. The mice were killed by dislocation of the neck, and the uterus was removed and weighed. Then the uterus was incubated in 10% phosphate (0.01 M) buffered formalin (pH 7.2) for at least 4 days with daily changes of formalin. Thereafter, the retained ^{125}I-radioactivity was measured with an autowell γ-counter. The results are expressed as the mean percentage of the radioactivity retained in the whole uterus to the injected radioactivity.

Effects of DHT at various doses, progesterone and oestradiol-17β on the retention of [^{125}I]IdUrd incorporated into the whole uterus and on the histology of the uterus

The experimental protocol was the same as that described above as far as the injection of [^{125}I]IdUrd. After the injection of [^{125}I]IdUrd, mice were divided into 7 groups and were daily injected at 6 p.m. with only the vehicle, DHT at a dose of 20, 50, 100 or 200 μg/mouse/day, progesterone (1 mg/mouse/day) and oestradiol-17β (1 μg/mouse/day), respectively. After 15 injections (on day 16), all the mice were killed, and [^{125}I]IdUrd retained in the whole uterus was determined. Thereafter, the uterus was embedded in paraffin. The transverse sections (5 μm thick) of the mid portion of the uterus were prepared and stained with hematoxylin and eosin.

Effects of flutamide on the retention of [^{125}I]IdUrd incorporated into the whole uterus

The experimental protocol was similar to that described above as far as the injection of [^{125}I]IdUrd. After the injection of [^{125}I]IdUrd, mice were divided into 6 groups and were daily injected at 6 p.m. with only the vehicle, flutamide (2 mg/mouse/day), DHT (100 μg/mouse/day), DHT (100 μg/mouse/day) plus flutamide (2 mg/mouse/day), progesterone (1 mg/mouse/day), and progesterone (1 mg/mouse/day) plus flutamide (2 mg/mouse/day), respectively. After 15 injections (on day 16), all the mice were killed, and [^{125}I]IdUrd retained in the whole uterus was determined.

Autoradiography

Six castrated female mice were daily injected with oestradiol-17β (1 μg/mouse/day) at 6 p.m. for 3 days, and at 8 a.m. on the day following the last injection of oestradiol-17β, all the mice were given an i.p. injection of 8 nmol/g body wt fluorodeoxyuridine. After 1 h, 2 μCi/g body wt [methyl-^{3}H]thymidine (86.4 Ci/mmol) was injected i.p. The mice were killed 4 h after the injection of [methyl-^{3}H]thymidine. The uterus was removed, soaked in 10% buffered formalin for 4 days with daily changes of formalin and embedded in paraffin. The transverse sections (5 μm thick) of the mid portion of the uterus were made, deparaffinized, dipped into Sakura NR-M2 emulsion, dried, and exposed for 21 days in a refrigerator (4°C). After development, the sections were stained with hematoxylin and eosin. About 1000 cells were counted under oil immersion and the percentage of the labelled cells was determined in the luminar and glandular epithelia and stroma [14]. The presence of more than 5 grains on the nucleus was regarded an indication of positive labelling.

Effect of DHT on the apoptotic index

Castrated female mice were daily injected with oestradiol-17β (1 μg/mouse/day) at 6 p.m. for 3 days. On the day following the last injection of oestradiol-17β (day 1), seven mice were killed and the uterus was removed. The remaining mice were divided into 2 groups and were daily injected with only the vehicle or DHT (100 μg/mouse/day) at 6 p.m. from day 1. Seven mice of each group were killed following 2, 5, 8, 11 and 14 injections (on days 3, 6, 9, 12 and 15) and the uterus was removed. All the uteri were fixed in 10% buffered formalin and embedded in paraffin. The transverse sections (5 μm thick) of the mid portion of the uterus were prepared and stained with hematoxylin and eosin. About 1000 cells were examined. Apoptotic cells were characterized by

the loss of contact with neighbouring cells, pyknosis and cytoplasmic condensation [3]. The percentage of apoptotic cells was determined as an apoptotic index.

Statistical analysis

The statistical significance ($P < 0.05$) was determined by the Student's *t*-test.

RESULTS

Effect of DHT on the retention of [^{125}I]IdUrd incorporated into the uterus

Castrated female mice which had been daily injected with oestradiol-17β (1 μg/mouse/day) for 3 days were injected with [^{125}I]IdUrd on the day following the last injection of oestradiol-17β (day 1), and the retention of [^{125}I]IdUrd incorporated into the whole uterus was examined after the daily treatment with only the vehicle or DHT (100 μg/mouse/day). Three injections of oestradiol-17β increased the uterine weight to about 53 mg (Fig. 1). However, when injected with the vehicle thereafter, the uterine weight decreased rapidly and was about 19 mg on day 16. The uterine weight decreased slightly on day 4 in spite of injections of DHT but the DHT injections gradually increased the uterine weight thereafter. The uterine weight on day 16 was about 75 mg as a result of the continued DHT injection.

The injection of only the vehicle resulted in a marked loss of the ^{125}I-radioactivity retained in the uterus until day 13 and there was no significant loss of the ^{125}I-radioactivity thereafter (Fig. 2). The injections of DHT did not completely prevent the loss of the ^{125}I-radioactivity but reduced it significantly.

When examined after 15 injections of DHT at various doses, the retention of [^{125}I]IdUrd incorporated in the whole uterus was dependent on doses (20–200 μg/mouse/day) of DHT (Fig. 3).

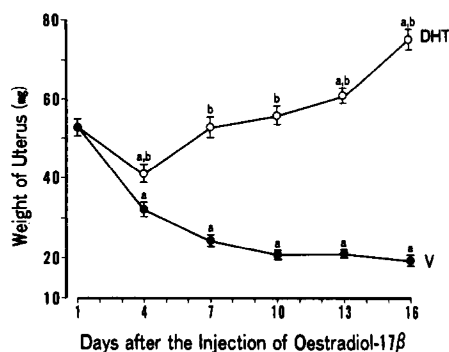

Fig. 1. Effect of DHT on the weight of the uterus. Castrated female mice were daily injected with oestradiol-17β (1 μg/mouse/day). After 3 injections of oestradiol-17β, all mice were injected with [^{125}I]IdUrd. Mice were divided into 2 groups and were injected daily with only vehicle (V, ●) or DHT (DHT, ○). DHT was injected at a dose of 100 μg/mouse/day. Day 1 is the day when the injection of vehicle or DHT was started. Each point represents the mean ± SE in 8 mice. $^{a}P < 0.05$, significant difference from the value on day 1. $^{b}P < 0.05$, significant difference from the value of the mice injected with only vehicle.

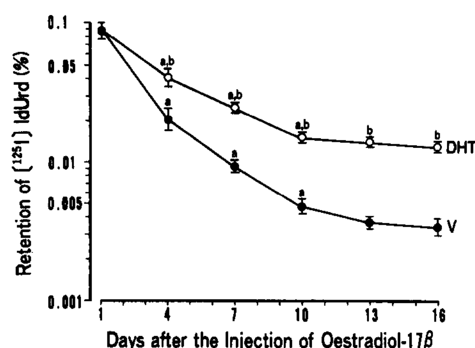

Fig. 2. Effect of DHT on the retention of [^{125}I]IdUrd incorporated into the whole uterus. The experimental design is shown in the legend of Fig. 1. The results of injections of only vehicle and DHT are indicated by V (●) and DHT (○), respectively. Each point represents the mean ± SE in 8 mice. $^{a}P < 0.05$, significant differences from the value of the preceding point. $^{b}P < 0.05$, significant difference from the value of mice injected with only vehicle.

In order to determine the type of cells whose DNA was labelled with ^{125}I, the labelling indices of luminal and glandular epithelial cells, and stromal cells were examined. [^{3}H]Thymidine was injected into the mice instead of [^{125}I]IdUrd and the labelling indices of uterine cells were determined. The labelling indices of luminal and glandular epithelial cells and stromal cells were 38.2 ± 1.3, 12.9 ± 1.2 and $6.4 \pm 0.2\%$ (mean ± SE, $n = 6$), respectively.

Effect of DHT on the apoptotic index of uterine cells

The effect of DHT on the uterine cell death was investigated morphologically. Castrated mice were daily injected with oestradiol-17β for 3 days. These mice were daily injected with only the vehicle or DHT (100 μg/mouse/day) from the day following the last injection of oestradiol-17β (day 1) and the apoptotic index of uterine cells was determined. The apoptotic

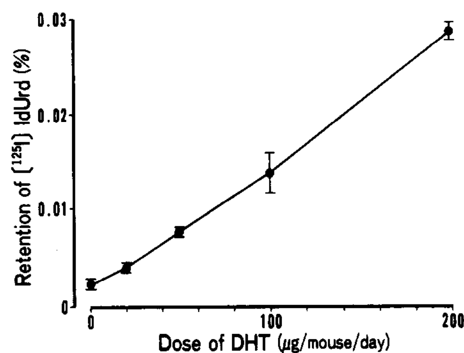

Fig. 3. Dose-dependent effect of DHT on the retention of [^{125}I]IdUrd incorporated into the whole uterus. Castrated female mice were daily injected with oestradiol-17β (1 μg/mouse/day). After 3 injections of oestradiol-17β, all mice were injected with [^{125}I]IdUrd. Mice were divided into 5 groups, and were daily injected with DHT at a dose of 0, 20, 50, 100 or 200 μg/mouse/day for 15 days. Mice were killed on the day following the last injection. Each point represents the mean ± SE in 7–8 mice.

Table 1. Effect of DHT on the apoptotic index of mouse uterine cells

Cell	Injection of DHT	Apoptotic index (%)[a]					
		Day 1	Day 3	Day 6	Day 9	Day 12	Day 15
Luminal epithelium	Yes		16.8 ± 3.37	0.2 ± 0.07	0.1 ± 0.04	<0.1	0.3 ± 0.08
	No	0.5 ± 0.11	11.0 ± 0.73	5.5 ± 0.56*	2.2 ± 0.20*	1.1 ± 0.28*	0.4 ± 0.13
Glandular epithelium	Yes		2.3 ± 1.26	0.4 ± 0.12	0.2 ± 0.10	0.3 ± 0.14	0.6 ± 0.08
	No	0.5 ± 0.09	9.0 ± 0.92*	5.6 ± 0.43*	2.7 ± 0.30*	2.2 ± 0.40*	2.2 ± 0.11*
Stroma	Yes		0.6 ± 0.17	0.2 ± 0.05	0.2 ± 0.05	0.1 ± 0.05	0.2 ± 0.03
	No	<0.1	0.7 ± 0.10	0.4 ± 0.04	0.2 ± 0.04	0.3 ± 0.03	0.2 ± 0.05

Castrated female mice were daily injected with oestradiol-17β (1 μg/mouse/day). After 3 injections of oestradiol-17β, mice were divided into 2 groups and injected daily with only the vehicle or DHT (100 μg/day). Day 1 is the day when the injection of vehicle or DHT was started. [a]Mean ± SE of values in 7 mice. *$P < 0.05$, significant difference from the value in mice injected with DHT by t-test.

indices of luminal and glandular epithelia and stroma were low on day 1 (Table 1). When the only the vehicle was injected, the apoptotic index of luminal epithelium markedly increased on day 3 and gradually decreased thereafter. Injections of DHT did not reduce the apoptotic index of luminal epithelium on day 3 but significantly decreased it on days 6, 9 and 12. The apoptotic index of glandular epithelium also increased markedly on day 3 when only the vehicle was injected. It decreased gradually thereafter but stayed relatively high even on day 15. Injections of DHT dramatically reduced the apoptotic indices on days 3–15. In contrast to the apoptotic indices of luminal and glandular epithelia, the apoptotic index of stroma was low even when only the vehicle was injected. Furthermore, the injections of DHT had no significant effect on the apoptotic index of stroma.

Effects of flutamide on the retention of [^{125}I]IdUrd incorporated into the whole uterus

The effects of the pure antiandrogen, flutamide on the action of DHT on the retention of [^{125}I]IdUrd and the uterine weight were examined. Injections of flutamide only, did not cause any effects on the retention of [^{125}I]IdUrd and the uterine weight (Table 2). However, flutamide completely abolished the effects of DHT on the retention of [^{125}I]IdUrd and the uterine weight. As previously reported [9], progesterone increased the retention of [^{125}I]IdUrd, and the effect of progesterone was not prevented by flutamide.

Effects of various steroids on the retention of [^{125}I]IdUrd incorporated into the whole uterus and on the histology of the uterus

The effects of progesterone and oestradiol-17β on the uterine cell death and histology were compared to those of DHT. Castrated female mice which had been daily injected with oestradiol-17β (1 μg/mouse/day) for 3 days were injected with [^{125}I]IdUrd and thereafter, these mice were daily injected with only the vehicle, DHT (100 μg/mouse/day), progesterone (1000 μg/mouse/day) or oestradiol-17β (1 μg/mouse/day) for 15 days (Table 3). These three steroids significantly increased the retention of [^{125}I]IdUrd incorporated into the whole uterus. Progesterone increased the retention of [^{125}I]IdUrd a little more than DHT, but oestradiol-17β increased it to a less extent compared to DHT or progesterone.

As shown in Fig. 4, luminal epithelial cells were atrophic, when only the vehicle was injected. Oestradiol-17β caused hypertrophy of luminal epithelial cells and transformed them into tall columnar cells. Progesterone and DHT caused similar changes in luminal epithelial cells. These cells had a vacuole under the nucleus and accumulated eosinophilic substance(s) above the nucleus.

Table 2. Effects of flutamide on the retention of [^{125}I]IdUrd incorporated into the whole uterus and the uterine weight

Injection of steroid or flutamide	No. of mice	Retention of [^{125}I]IdUrd ($\times 10^{-3}$%)[a]	Uterine weight (mg)[a]
No	7	3.4 ± 0.3	16.6 ± 0.6
F	6	4.4 ± 0.9	15.8 ± 0.7
DHT	7	18.6 ± 1.8*†	73.4 ± 3.5*†
DHT + F	7	4.6 ± 0.3	18.1 ± 0.7
Progesterone	7	26.3 ± 1.4*	31.2 ± 0.8*
Progesterone + F	7	23.9 ± 1.3*	32.9 ± 1.0*

Castrated female mice were daily injected with oestradiol-17β (1 μg/mouse/day). After 3 injections of oestradiol-17β, all mice were injected with [^{125}I]IdUrd. Mice were divided into 6 groups and were daily injected with only the vehicle, flutamide (F), DHT, DHT plus flutamide, progesterone or progesterone plus flutamide for 15 days. Flutamide, DHT, and progesterone were injected at a dose of 2 mg, 100 μg and 1 mg/mouse/day, respectively. [a]Mean ± SE. *$P < 0.05$, significant difference from the value in mice injected with only the vehicle or flutamide by t-test. †$P < 0.05$, significant difference from the value in mice injected with DHT plus flutamide by t-test.

DISCUSSION

The effect of androgen on the death of mouse uterine cells was investigated by measuring the

Table 3. Effects of various steroids on the retention of [^{125}I]IdUrd incorporated into the whole uterus

Injection of steroid	Dose (μg/mouse/day)	No. of mice	Retention of [^{125}I]IdUrd ($\times 10^{-3}$%)[a]
No	—	8	2.8 ± 0.4
DHT	100	8	12.7 ± 1.3*
Progesterone	1000	9	17.6 ± 2.0*
Oestradiol-17β	1	7	8.4 ± 0.4*†

Castrated female mice were daily injected with oestradiol-17β (1 μg/mouse/day). After 3 injections of oestradiol-17β, all mice were injected with [^{125}I]IdUrd. Mice were divided into 4 groups and were daily injected with only the vehicle, DHT, progesterone or oestradiol-17β for 15 days. [a]Mean ± SE. *$P < 0.05$, significant difference from the value in mice injected with only the vehicle by t-test. †$P < 0.05$, significant difference from the value in mice injected with DHT or progesterone by t-test.

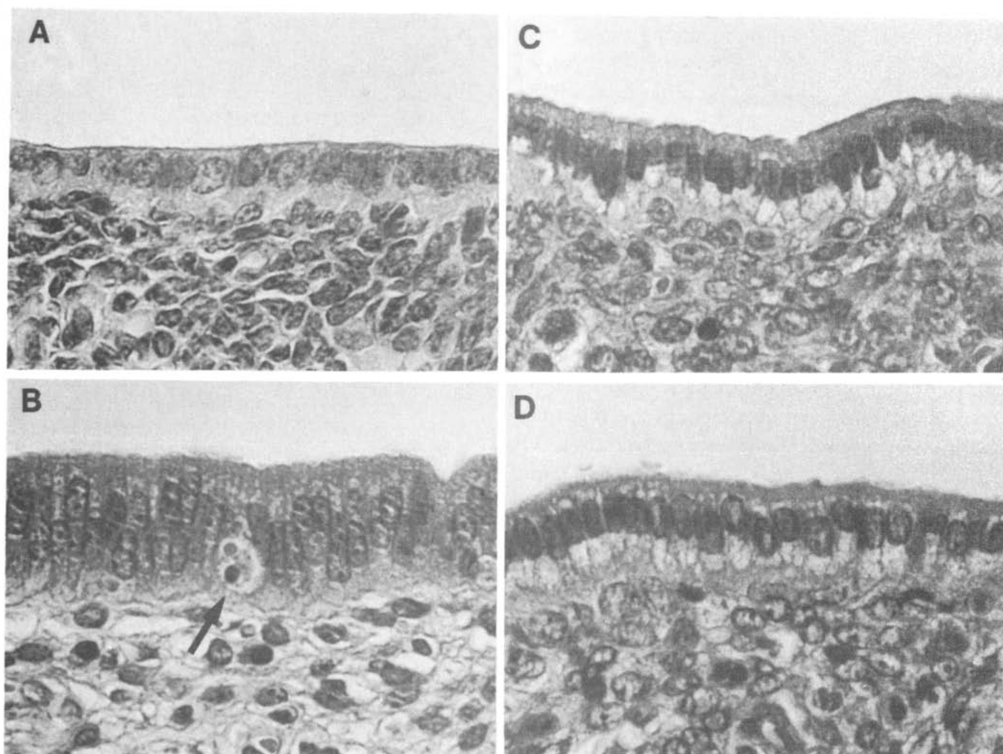

Fig. 4. Effects of oestrogen, progesterone and DHT on the histology of luminal epithelium. The histology of the uterus of mice described in Table 3 was examined. Hematoxylin and eosin. ×600. (A) Injections of vehicle caused atrophy of luminal epithelial cells. (B) Injections of oestradiol-17β transformed luminal epithelial cells into tall columnar cells. An arrow indicates an apoptotic cell. (C) Injections of progesterone resulted in luminal epithelial cells having a vacuole under the nucleus and an eosinophilic substance above it. (D) Injections of DHT resulted in luminal epithelial cells with a similar histology to those treated with progesterone.

retention of [^{125}I]IdUrd incorporated into the whole uterus. As we have shown, more than 90% of the ^{125}I-radioactivity incorporated into the uterus was retained in the fraction of DNA, when the radioactivities in the fractions of RNA, DNA and protein were examined [15]. Furthermore, the autoradiographic study showed that the injection of oestradiol-17β resulted in lebelling of cells predominantly in the luminal and glandular epithelia. Thus, examination with [^{125}I]IdUrd seems to be valid for the estimation of the cell death of the uterine epithelium. Finn and Publicover [8] have adopted a similar method to examine the effect of oestrogen on the uterine cell death, although they used [^{3}H]thymidine instead of [^{125}I]IdUrd.

The effect of androgen on the cell death was also examined by the morphological method. The results obtained by this method were in good agreement with those obtained by the method with [^{125}I]IdUrd. Furthermore, it became evident that the cell loss after the depletion of oestrogen in the uterus occurs mainly in epithelium and that DHT prevents the cell death of epithelium. In consistency with our results, it has been shown that the cell loss in the uterus occurs mainly in epithelium after the ovariectomy of pseudopregnant rabbits [6] and only the steroid-regulated cell loss of uterine epithelium has been reported in some species of animals [2, 4, 7, 9].

Previous studies have shown that androgen binds to the oestrogen receptor and that androgen could act through the oestrogen receptor [16–18]. In the present study, the effect of DHT on the retention of [^{125}I]IdUrd was abolished by the pure antiandrogen, flutamide. Thus, it seems that androgen protects the uterine epithelial cells from death through not the oestrogen receptor but the androgen receptor. On the other hand, the effect of progesterone on the retention of [^{125}I]IdUrd was not prevented by flutamide. This implies that androgen and progesterone work through different receptor systems.

The luminal epithelium treated with oestrogen manifested apparently different histology compared to that treated with progesterone and DHT. On the other hand, the histology of the luminal epithelium treated with progesterone was similar to that treated with DHT. Progesterone, oestrogen and DHT reduced the uterine cell loss which occurred mainly in the epithelium. Thus, this result may suggest that the mechanism of the inhibitory action of androgen on the cell death may be similar to that of progesterone but different from that of oestrogen.

The cell loss of the mouse uterine epithelium primed with oestrogen was reduced by oestrogen, progesterone and androgen. The preventive action of oestrogen on the cell loss of uterine epithelium primed with oestrogen has also been reported in rats [7, 8]. However, Nawaz *et al.*[4] reported that oestrogen did not maintain the uterine epithelial cells in pseudopregnant rabbits after castration although progesterone did, whereas they did not examine the effect of oestrogen or progesterone on the uterine epithelium of a castrated rabbit primed with oestrogen. The uterine epithelium of a pseudopregnant rabbit is exposed to both oestrogen and progesterone. Thus, the failure in prevention of uterine cell loss by oestrogen in castrated pseudopregnant rabbits may suggest that the actons of various steroids on the uterine cell death may differ in the uterine epithelium primed with only oestrogen and in that primed with both oestrogen and progesterone. If it is not the case, it may suggest that the actions of various steroids on the cell death of uterine epithelium may differ among animal species. These two possibilities should be further evaluated by investigating the preventive action of various steroids on the cell death of uterine epithelia primed with only oestrogen and with both oestrogen and progesterone in many species.

In conclusion, the present study indicates that androgen prevents the cell death of the epithelium in the mouse uterus and again emphasizes that the preventive action of cell death is one of the important actions of steroids.

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