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Growth-stimulating effect of dienogest, a synthetic steroid, on rodent, canine, and primate mammary glands

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Abstract

We observed hyperplasia of the mammary gland in female beagle dogs, but not in female rats and monkeys, in 91-day toxicity studies on dienogest. In order to elucidate a possible mechanism for its development and to account for this species difference, we determined the plasma level of growth hormone (GH) in dogs, rats, and monkeys treated orally with dienogest for 91 days. As a result, dogs with mammary hyperplasia showed a prominent, dose-dependent increase in their GH level; and, contrarily, rats and monkeys without the hyperplasia of this organ failed to show any such increase. These results were supported by evidence from immunohistochemical and morphometric analysis of the pituitary gland. In addition, dienogest and medroxyprogesterone acetate (MPA) stimulated the growth of canine mammary epithelial cells in the presence of estradiol in vitro, but had no effect on rat and human mammary epithelial cells incubated under the same conditions. In conclusion, dienogest with progestational activity caused proliferation of the mammary gland in beagle dogs by increasing the secretion of GH, as do other progestational compounds. This change may be partially dependent on the direct effect of the drug.

Keywords: Dienogest; Dogs; Growth hormone; Mammary gland; Synthetic steroid

1. Introduction

Dienogest, (17α-cyanomethyl-17β-hydroxy-estra-4,9-dien-3-one; Fig. 1, upper), is an orally active synthetic steroid with prominent progestational activity that is used for contraception and is currently being studied for the possible treatment of endometriosis (Katsuki et al., 1997, 1998). In repeated toxicity studies on dienogest, we observed that beagle dogs showed proliferation of mammary epithelium, whereas rats and rhesus monkeys failed to exhibit evidence of such proliferation. Thus, this proliferative change was specific to dogs. The purpose of the present study was to elucidate the mechanism responsible for the proliferation of canine mammary epithelium induced by dienogest.

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2. Materials and methods

2.1. Test materials

In the 91-day toxicity study conducted previously, the test drug, dienogest (Jena Pharm, Jena, Germany), was suspended in 0.5% carboxymethylcellulose (Wako Pure Chemical, Osaka, Japan) and orally administered to rats, dogs, and monkeys. In this in vitro study, dienogest, medroxyprogesterone acetate (MPA; Fig. 1, lower, Sigma Chemical, St. Louis, MO), and 17 beta-estradiol (Sigma Chemical) were dissolved in dimethylsulfoxide (DMSO, Wako Pure Chemical) and then sterilized by passage through a filter (0.2 μm in diameter, Advantec, Tokyo, Japan).

2.2. Animals

Six-week-old female Slc: Wistar rats (Japan SLC, Hamamatsu, Japan), 9 or 10-month-old female beagle dogs (White Eagle Laboratories, Doylestown, PA), and 3 or 4-year-old female rhesus monkeys bred in purpose (CSK Research Park, Suwa, Japan) were subjected to rat, dog, or monkey 91-day toxicity study, respectively. The rats, dogs, and monkeys were individually housed in aluminium cages (170W × 260D × 180H mm), stainless steel cages (780W × 800D × 960H mm), and stainless steel cages (600W × 700D × 700H mm), respectively, in rooms, a separate one for each species, kept at a controlled temperature (23 ± 2°C) and relative humidity (55 ± 15%) and having a 12-h light/12-h dark cycle. The rats, dogs, and monkeys were given a certified solid diet, FR-2 (Funabashi Farm, Funabashi, Japan), Lab Diet #4360 (Purina Japan, Tokyo, Japan), and Primate Diet #5048 (Purina Japan), respectively, and free access to water during the experimental period. At the start of dosing, the ranges of body weight of the rats, dogs, and monkeys were 110–122 g, 7.7–10.9 kg, and 3.5–4.9 kg, respectively. The guidelines of Mochida Pharmaceutical were followed for the care and use of the animals in these studies. Based on the results of our 14-day dose-determining study on rats, on those of the 6-month toxicity study on dogs reported by Hoffmann et al. (1983), and on those of our 91-day toxicity study on dogs, we set up four, three, and three doses for the rats, dogs, and monkeys, respectively, to clarify toxic and nontoxic dose levels in each species. The rats, dogs, and monkeys were treated orally for 91 days with 0 (vehicle alone), 0.3, 1, 3, or 30 mg/kg/day of dienogest; 0, 0.03, 0.3, or 3 mg/kg/day; and 0, 0.1, 1, or 10 mg/kg/day, respectively. Control animals were given vehicle (0.5% carboxymethylcellulose) alone. As a result, the nontoxic dose level for rats, dogs, and monkeys was 3, 0.03 (except mammary gland proliferation), and 1 mg/kg/day, respectively. The above-mentioned 91-day toxicity studies were conducted in accordance with the guideline on Good Laboratory Practice published by the Japanese Ministry of Health and Welfare.

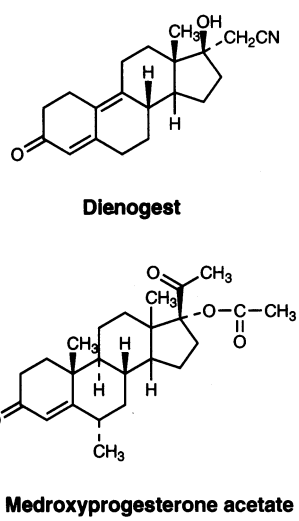

Fig. 1. Chemical structures of dienogest and medroxyprogesterone acetate.

2.3. Plasma samples

Plasma samples were obtained from the rats (n = 5 for each dose), dogs ($n = 3$ or 4), and monkeys ($n = 3$ or 5) treated orally with dienogest or the vehicle for 91 days at the above-mentioned dose levels for each species. The plasma samples were used for determination of their growth hormone (GH) level and were stored at −80°C before being assayed.

2.4. Measurement of plasma GH levels

Plasma GH levels were measured by a time resolved-fluoroimmunoassay (Hemmilä, 1988) using the DELFIA system (Pharmacia Biotech, Uppsala, Sweden). Briefly, 100 μl of goat anti-rabbit IgG antibody (Funakoshi, Tokyo, Japan) diluted 1:100 with phosphate-buffered saline (PBS) was added to each well of a 96-well microplate (NUNC, Roskilde, Denmark) and incubated for 30 min at 37°C. After discarding of the solution, each well was washed with PBS containing 0.05% Tween 20. Then, 100 μl of PBS containing 0.1% bovine serum albumin (Sigma Chemical) was added to each well, and incubation was carried out for 15 min at room temperature. After removal of the solution, each well was washed again as described above. Finally, 50 μl of rabbit anti-rat GH antibody (Cosmobio, Tokyo, Japan), rabbit anti-dog GH antibody (Shibayagi, Gunma, Japan), or rabbit anti-human GH antibody (Cosmobio) solution was added simultaneously with 50 μl of europium-labeled rat, dog, or human GH (Cosmobio; purity; 90, 95, and 98%, respectively) solution and 50 μl of plasma samples from rats, dogs, or monkeys to each well, and incubation was then carried out for 30 min at 37°C. After discarding of the solution, each well was washed with DELFIA washing solution. Then, 100 μl of enhancement solution [0.1 M acetate buffer (pH 3.2) containing 15 μM α-2-naphtoyltrifluoro acetate, 50 μM tricyclphosphenoxide, and 0.1% Triton-X 100] was added, and incubation was carried out for 5 min at 25°C. The fluorescence of europium was measured with a DELFIA fluorometer, and the GH concentration was calculated from the standard curve previously prepared. The detection limit of GH level was about 100 pg/ml for a 50 μl plasma sample from rats, dogs, or monkeys.

2.5. Mammary glands and pituitary glands

On the day after the last dosing, the animals were killed by exanguination under anesthesia with pentobarbital sodium; and then their mammary glands, pituitary glands, etc. were removed. The mammary glands obtained from rats ($n = 10$) given dienogest at 0 or 30 mg/kg/day, dogs ($n = 3$) treated with the drug at 0, 0.03, 0.3, or 3 mg/kg/day, and monkeys ($n = 3$) given it at 0 or 10 mg/kg/day were routinely fixed in 10% phosphate-buffered formalin, embedded in paraffin, cut at a thickness of 3 μm, stained with hematoxylin and eosin, and examined under a light microscope. The pituitary glands obtained from the above-mentioned animals were stained immunohistochemically for GH and morphometrical analysis as described below.

2.6. Immunohistochemical analysis of GH

Deparaffinized sections of the pituitary glands were treated with 3% H_2O_2 to inactivate endogenous peroxidase, rinsed in PBS, and then incubated with blocking serum (goat serum, Cosmobio) for 20 min at room temperature. Next, the sections from rats, dogs, and monkeys were treated with rabbit anti-rat GH, rabbit anti-dog GH, and rabbit anti-monkey GH antibodies (Biogenesis, Poole, UK), respectively. After having been washed with PBS, the sections were next incubated with a biotinylated secondary antibody (goat anti-rabbit IgG) for 30 min at room temperature. After another washing with PBS, the sections were incubated with streptoavidin-peroxidase and again washed with PBS. Finally, the sections were treated with 3, 3′-diaminobenzidine-H_2O_2 substrate medium, rinsed in tap water, and counterstained with hematoxylin. The size of the cells positive for GH staining was determined morphometrically with an image analyzer (LUZEX III, Nireco, Tokyo, Japan).

2.7. In vitro effects of dienogest and medroxyprogesterone acetate (MPA) on mammary epithelial cells from rats, dogs, and humans

2.7.1. Preparation of rat and dog mammary epithelial cells

On day 16 or 17 of gestation, pregnant Slc: Wistar rats were killed by exanguination under anesthesia with pentobarbital sodium (25 mg/kg, i.p.). Then, the mammary glands were removed.

Mammary tissues of lactating beagle dog dams belonging to CSK Research Park (Suwa, Japan) were obtained by biopsy under anesthesia with pentobarbital sodium (30 mg/kg, i.p.). After removal of the vessels and adipose tissues from the mammary tissues, the latter were washed with sterilized PBS. The tissues were next minced with a surgical knife, immediately dispersed in collagenase (Nitta Gelatin, Tokyo, Japan) solution (6000 units/ml) for 10 min at 37°C with shaking (10 times/min), and then washed in MEM medium (Nissui Pharmaceutical, Tokyo, Japan). Thereafter, the cells were incubated in deoxyribonuclease (Sigma Chemical) solution (1000 units/ml) for 10 min at 37°C with shaking (10 times/min), washed in MEM medium, and suspended in the medium at a concentration of 10^4/ml. Aliquots of the cell suspension (1000 cells in 0.1 ml) were distributed into several plastic plates (Primaria, Falcon) coated with collagen type-IV and basement element (Nitta Gelatin) and containing 0.9 ml of MEM medium supplemented with fetuin (1 mg/ml, Sigma Chemical), insulin (5 μg/ml, Sigma Chemical), transferrin (5 μg/ml, Sigma Chemical), fibroblast growth factor (100 ng/ml, Funakoshi), and epidermal growth factor (10 ng/ml, Funakoshi), and then incubated at 37°C under an atmosphere of 5% CO_2 and 95% air.

2.7.2. Human mammary epithelial cells

Normal human mammary epithelial cells (Mammary pack) were purchased from Kurabo (Tokyo, Japan). The cells were suspended in the above-mentioned medium for their proliferation. Aliquots of the cell suspension (1000 cells in 0.1 ml) were distributed into several plastic plates (Falcon) coated with collagen type-IV and basement element (Nitta Gelatin) and incubated at 37°C under an atmosphere of 5% CO_2 and 95% air.

2.7.3. Treatment with dienogest or MPA

Before the cells had become confluent, they were isolated with 0.25% trypsin solution in PBS and used for an in vitro study. An aliquot of the cell suspension (1000 cells) was distributed into a plastic plate (Falcon) incubated for 60 min at 37°C, and then cultured with DMSO as a vehicle control or with dienogest (3.2×10^{-11}, 3.2×10^{-10}, 3.2×10^{-9}, or 3.2×10^{-8} M as the final concentration) or MPA (2.6×10^{-11}, 2.6×10^{-10}, 2.6×10^{-9}, or 2.6×10^{-8} M as the final concentration) in the absence or presence of estradiol (3.7×10^{-11}, 3.7×10^{-10}, or 3.7×10^{-9} M as the final concentration) for 7 days at 37°C under an atmosphere of 5% CO_2 and 95% air. We selected these doses based on the results of a previous experiment in which dienogest suppressed the proliferation of rat endometrial cells in the presence of estradiol (Katsuki et al., 1998). After the culture period, the cells were stained with trypan blue (Wako Pure Chemical); and the number of viable cells ($\times 10^3$/culture) was then counted under an inverted microscope.

2.8. Pharmacokinetics of dienogest in rats, dogs, and monkeys

Five-week-old female Slc: Wistar rats (Japan SLC), 2-year-old female beagle dogs (White Eagle Laboratories), and 5- or 6-year-old female rhesus monkeys bred in purpose (CSK Research Park) were used, 5, 3, and 3 animals, respectively, for this pharmacokinetic study. At 0.25, 0.5, 1, 2, 4, 6, and 24 h after oral administration of dienogest to the rats at a dose of 10 mg/kg corresponding to the high doses in the 91-day toxicity study, to the dogs at a dose of 0.03 mg/kg, which caused apparently mammary gland hyperplasia, and to the monkeys at a dose of 10 mg/kg, equal to the high dose in the 91-day toxicity study, heparinized blood samples were taken without anesthesia. The samples were centrifuged at $2200 \times g$ for 15 min at 4°C to isolate the plasma for determination of plasma dienogest levels. To the plasma sample (100 μl), 10 μl of ethanol and 10 μl of 17α-ethynylestradiol solution (20 μg/ml) as an internal standard were added. Then, 4.9 ml of 0.01 M phosphate-buffered saline (PBS, pH 7.4) containing 0.25% (v/v) sodium heparin was added to the mixture, vortex-mixed, and centrifuged at $2200 \times g$ for 10 min at room temperature to isolate the supernatant. The supernatant was

loaded onto a solid-phase extraction column; and the column washed successively with PBS, water, 20% acetonitrile, and 70% ethanol; and the drug and internal standard were eluted with 90% methanol. After the extract had been evaporated to dryness, the residue was dissolved in 120 μl of HPLC eluent [20 mM PBS (pH 7.0): acetonitrile = 60:40 (v/v)], and filtered through a membrane filter (0.45-μm pore size). A 50-μl aliquot of the filtrate was injected onto a HPLC column (Inertsil ODS-2, 4.6 $\phi \times 250$ mm). The compounds were separated on the column by use of a mobile phase consisting of 20 mM PBS (pH 7.0) and acetonitrile (60:40, v/v) and detected with a UV detector at wavelengths of 300 nm (0–12 min) and 280 nm (12–25 min). The peak height of dienogest relative to that of 17α-ethynylestradiol was calculated, and then the concentration of dienogest was determined from a calibration curve (10–2000 ng/ml) prepared previously by adding known amounts of dienogest to control plasma.

2.9. Statistical analysis

The results of each experiment were expressed as the mean ± standard deviation (SD). After analysis of variance, Dunnett's test was used to analyze statistically significant differences between the groups by the multicomparison procedure using SAS software (version 6, SAS Institute, Cary, NC). A statistically significant difference was defined at $P < 0.05$.

3. Results

3.1. Plasma levels of GH in female rats, dogs, and monkeys (Fig. 2)

In rats treated orally with dienogest (0.3, 1, 3, or 30 mg/kg) for 91 days, their plasma levels of GH were similar to those of the control animals. In contrast, in dogs treated orally with the drug (0.03, 0.3, or 3 mg/kg) for 91 days, their GH levels increased significantly in a dose-dependent fashion. The values at doses of 0.03, 0.3, and 3 mg/kg were ≈4, 9, and 18 times, respectively, greater than the control value. In monkeys treated orally with dienogest (0.1, 1, or 10 mg/kg) for 91 days, their GH levels were similar to those of the control animals.

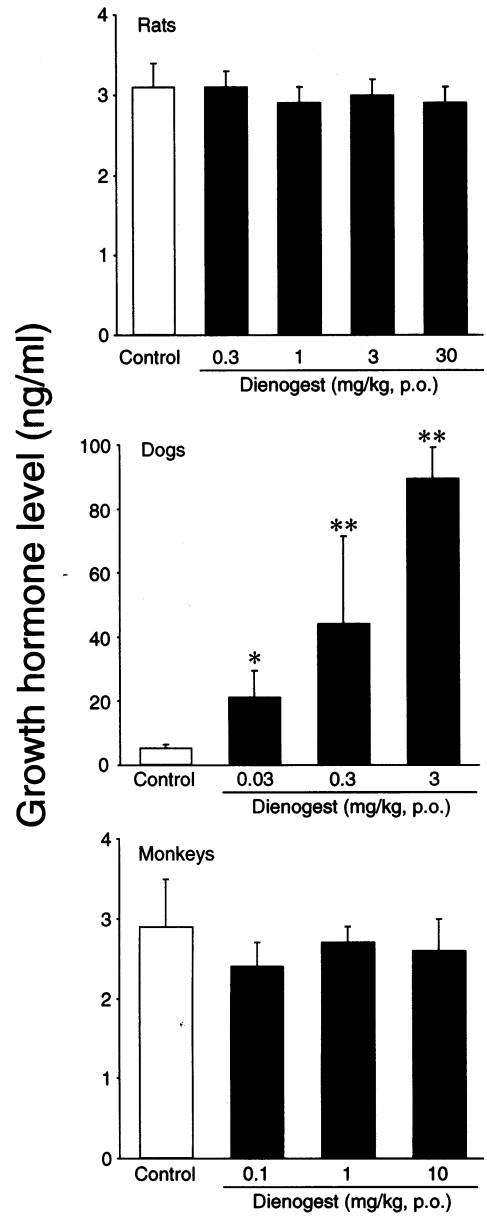

Fig. 2. Changes in plasma levels of growth hormone in female rats, dogs, and monkeys treated orally with dienogest for 91 days. Each column represents the mean ± SD ($n = 5$ for rats, $n = 3$ or 4 for dogs, and $n = 3$ or 5 for monkeys). Data were analyzed by analysis of variance and Dunnett's test. Significant differences from the control: $^{*}P < 0.05$, $^{**}P < 0.01$.

3.2. Histopathology, immunohistochemistry, and morphometry

The mammary glands from female rats and monkeys treated with dienogest for 91 days showed no abnormalities. In contrast, the mammary glands from female dogs treated with dienogest for 91 days showed dose-dependent proliferation of their mammary glands. Especially, the dogs (Fig. 3) given 3 mg/kg showed severe hyperplasia of alveoli and a large number of vacuoles in the alveolar cells. This histology resembled that seen during the lactation period. The cells producing GH in the pituitary glands from dienogest-treated rats (Fig. 4, B) and monkeys (Fig. 4, F) were histologically similar to

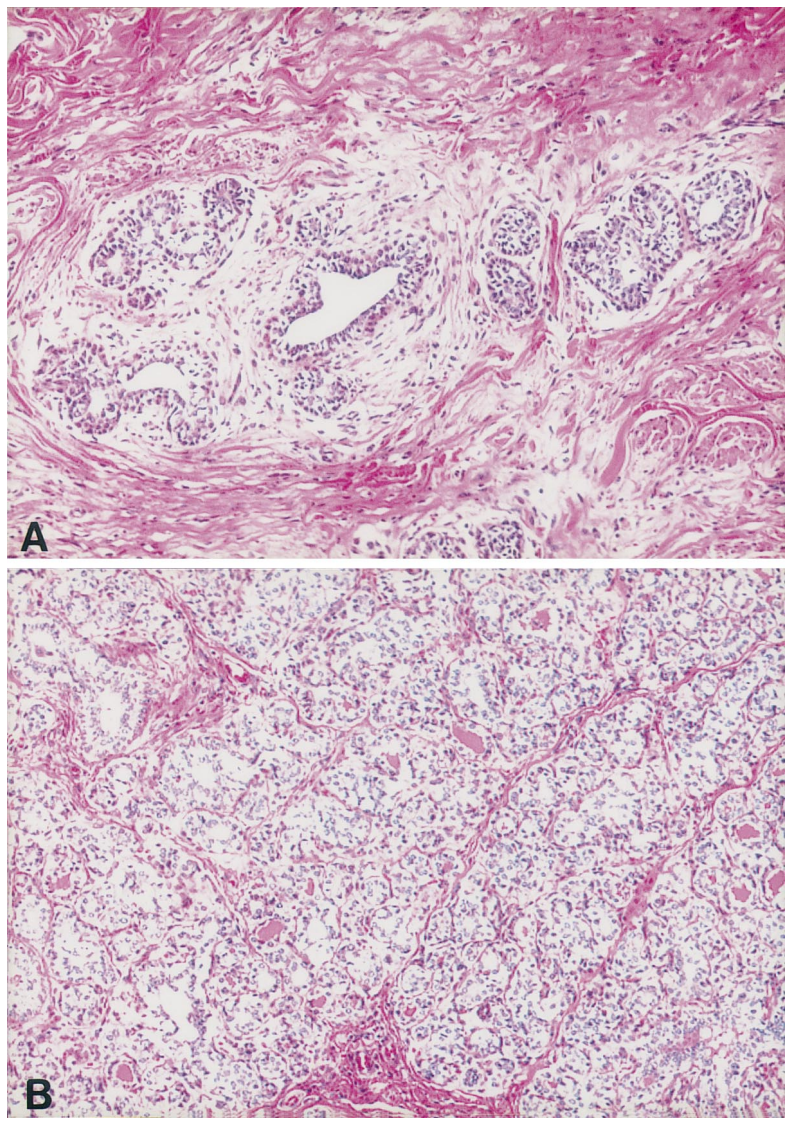

Fig. 3. Mammary glands from dogs treated orally with dienogest or vehicle for 91 days. A and B: Hyperplasia of acini is noticeable in the mammary gland from a dienogest (3 mg/kg) -treated dog (B) when this gland is compared with that from a vehicle-treated one (A). Acini are densely arranged in the mammary gland and the amount of interstitial tissues has decreased in the drug-treated gland. H&E stain, × 150.

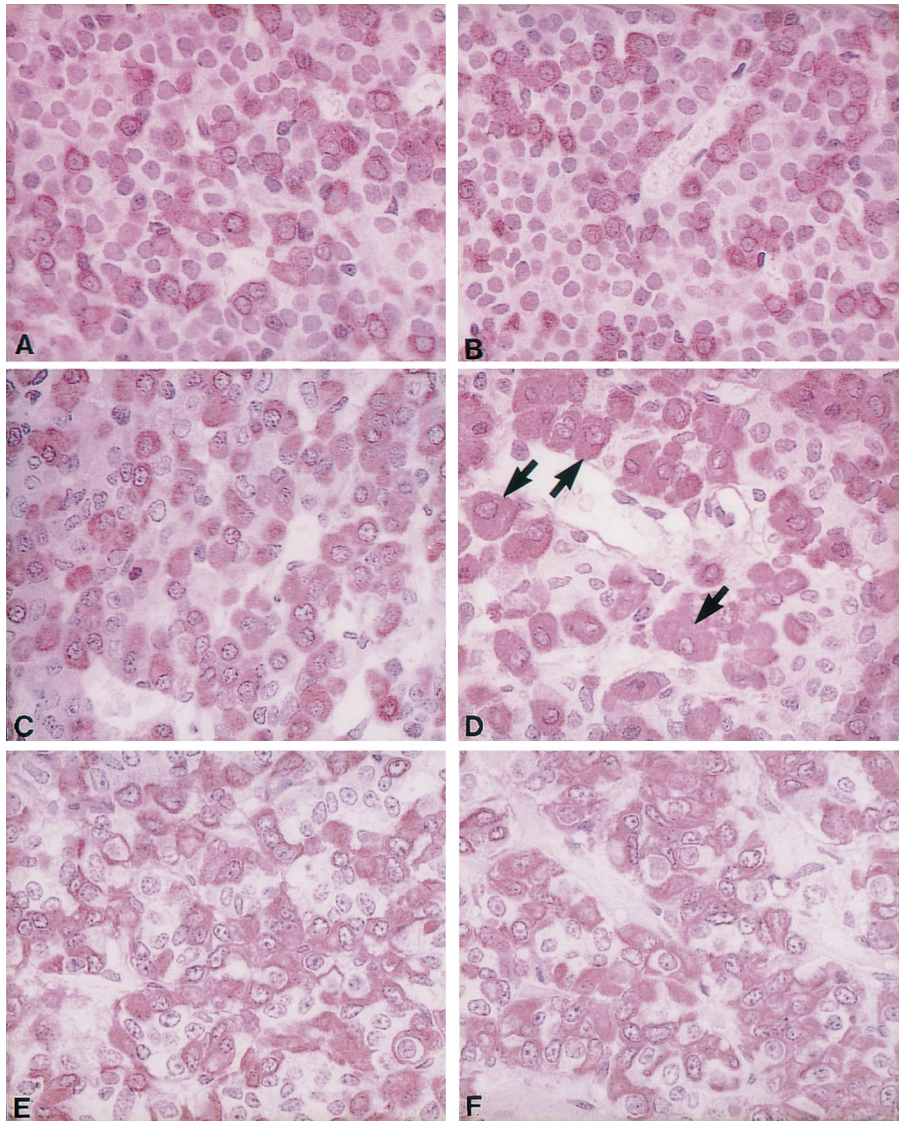

Fig. 4. Growth hormone immunostaining in the pituitary glands from rats (A and B), dogs (C and D), and monkeys (E and F) treated orally with dienogest or vehicle for 91 days. A and B: No differences are seen between a dienogest (30 mg/kg) -treated rat (B) and a vehicle-treated one (A). C and D: Slight hypertrophy of GH-positive cells (arrows) is noticeable in the pituitary gland from a dienogest (3 mg/kg) -treated dog (D) as compared with the size of these cells from a vehicle-treated one (C). GH-positive cells in D have a large amount of cytoplasm. E and F: No differences are seen between a dienogest (10 mg/kg) -treated monkey (F) and a vehicle-treated one (E). Immunohistochemical staining with anti-GH antibody, × 150.

those of the control animals (Fig. 4, A and E, respectively). In contrast, the pituitary glands from dienogest-treated dogs (Fig. 4, D) showed slight hypertrophy as compared with those from control dogs (Fig. 4, C). In addition, by morphometric analysis, the GH-positive cells in the pituitary glands from dienogest-treated dogs were dose-dependently larger in size than those of the control animals; and at 3 mg/kg the GH-positive cells were significantly larger than the control ones (Fig. 5).

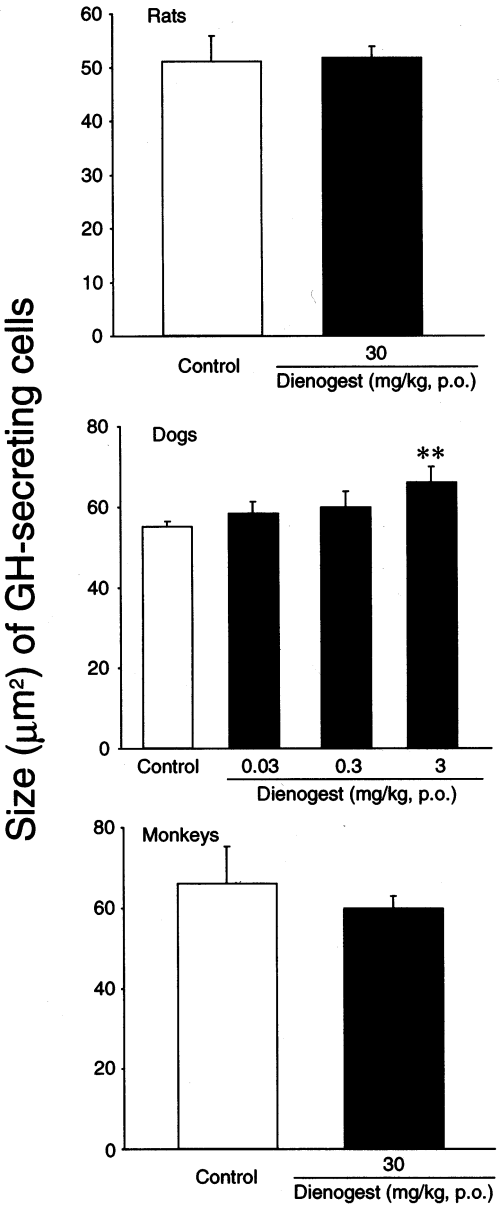

Fig. 5. Changes in size of growth hormone (GH) -secreting cells in the pituitary glands from rats, dogs, and monkeys treated orally with dienogest for 91 days. Each column represents the mean ± SD ($n = 10$ for rats, and $n = 3$ for dogs and monkeys). Data were analyzed by analysis of variance and Dunnett's test. A significant difference from the control: $^{**}P < 0.01$.

3.3. In vitro effects of dienogest and MPA on mammary epithelial cells from female rats, dogs, and humans (Table 1)

Dienogest (3.2×10^{-9} and 3.2×10^{-8} M) and MPA (2.6×10^{-9} and 2.6×10^{-8} M) had no effect on rat or human mammary epithelial cell growth in the presence or absence of estradiol. In contrast, dienogest (3.2×10^{-9} M or more) and MPA (2.6×10^{-10} M or more) stimulated slightly, but significantly, dog mammary epithelial cell growth in the presence of estradiol but did not do so in the absence of the hormone. Dienogest was equal to MPA in stimulative potency. Estradiol stimulated the growth of cells from all three species in a concentration-dependent manner.

3.4. Pharmacokinetics of dienogest in rats, dogs, and monkeys

Plasma concentration-time curves for rats, dogs, and monkeys after a single oral administration of dienogest are shown in Fig. 6. The relevant pharmacokinetic parameters for each species are shown in Table 2. The Cmax value for monkeys was about 3.6 times greater than that for rats; and AUC value for the former was ≈ 20.9 times greater than that for the latter. Therefore, systemic availability of dienogest was greater in monkeys than in rats. On the other hand, in dogs given 0.03 mg/kg, which showed a significantly increased secretion of growth hormone, Cmax and AUC values were about 1/100 of those in monkeys. Based on the dose used for each species, these results suggest that the dogs may have the greatest systemic availability of dienogest.

4. Discussion

Macroscopic mastoplasia was observed in the mammary tissue of female beagle dogs after 2 or 3 weeks of treatment in a 91-day toxicity study on dienogest at oral doses of 0.03, 0.3, and 3 mg/kg/day; but this change was not augmented by increasing the duration of exposure or by increasing the dose. The increase in mammary gland size was equal or slightly larger than that dependent on the

Table 1
Effects of dienogest and medroxyprogesterone acetate (MPA) in the absence or presence of 17 β-estradiol on growth of rat, dog, and human mammary epithelial cells in vitro[a]

Species	Drug	Concentration (M)	Vehicle	Estradiol (M)		
				3.7×10^{-11}	3.7×10^{-10}	3.7×10^{-9}
Rat	Control	–	3.8[b]	4.5	5.4	5.3
	Dienogest	3.2×10^{-9}	3.8	4.5	5.4	5.3
	Dienogest	3.2×10^{-8}	3.8	4.5	5.4	5.3
	MPA	2.6×10^{-9}	3.8	4.5	5.4	5.3
	MPA	2.6×10^{-8}	3.8	4.5	5.4	5.3
Dog	Control	–	3.9	4.7	5.9	5.9
	Dienogest	3.2×10^{-11}	3.9	4.7	5.9	6.0
	Dienogest	3.2×10^{-10}	3.9	4.7	6.0	6.4
	Dienogest	3.2×10^{-9}	3.9	4.8	6.3*	6.5**
	Dienogest	3.2×10^{-8}	3.9	4.8	6.7**	6.5**
	MPA	2.6×10^{-11}	3.9	4.7	5.9	5.9
	MPA	2.6×10^{-10}	3.9	4.7	5.9	6.4*
	MPA	2.6×10^{-9}	3.9	4.7	6.4**	6.6**
	MPA	2.6×10^{-8}	3.9	4.9*	6.7**	6.6**
Human	Control	–	2.3	3.2	4.2	4.6
	Dienogest	3.2×10^{-9}	2.3	3.3	4.1	4.6
	Dienogest	3.2×10^{-8}	2.3	3.2	4.1	4.6
	MPA	2.6×10^{-9}	2.3	3.2	4.1	4.5
	MPA	2.6×10^{-8}	2.3	3.2	4.2	4.6

[a] Each value represents the mean ($n=3$). Standard deviations were from 0.0 to 0.2. Data were analyzed by analysis of variance and Dunnett's test. Significant differences from the control: *$P<0.05$, **$P<0.01$. Significant differences between the vehicle and all estradiol treatments were observed at $P<0.01$.

[b] The total number of viable cells ($\times10^3$) per culture.

menstrual cycle (unpublished data). Histopathological examination revealed that the mammary gland proliferation occurred in a dose-dependent manner. On the other hand, there was no mastoplasia in mammary epithelia of female rats or monkeys in the 91-day toxicity studies on dienogest at oral doses of 0.3, 1, 3, and 30 mg/kg/day for the former and 0.1, 1, and 10 mg/kg/day for the latter. Dogs given 0.03 mg/kg, which induced hypermastia in the 91-day toxicity study, showed lower values for pharmacokinetic parameters (Cmax and AUC values) than those in rats and monkeys given 10 mg/kg. Based on the results of pharmacokinetics, these results show a clear species difference in dienogest-induced growth of the mammary gland. Neumann (1991) reported evidence for progestogen-induced proliferation of the mammary gland in female dogs: (1) The mammary gland in dogs treated with progestogens for a long-term period reached the same size as that seen during lactation; (2) Dog pituitary gland showed a prominent increase in the number of basophilic cells positive for paraldehydefuchsin staining, and these cells were immunohistochemically equivalent to the cells producing GH; (3) Serum GH level increased; (4) Canine GH was a prominent impetus for mammary gland enlargement and lactation; and (5) Mammotropic effects of progestogens were not observed in hypophysectomized dogs treated with these compounds. In addition, in MPA-treated dogs, an increased serum level of GH and development of mammary nodes were reported by Concannon et al. (1981), and an increase in the former was indicated by McCann et al. (1987). Furthermore, Gräf and El Etreby (1979) reported these same changes in dogs treated with cyproterone acetate. These results suggest that progestational compounds have no direct proliferation-stimulating effect on the canine mammary

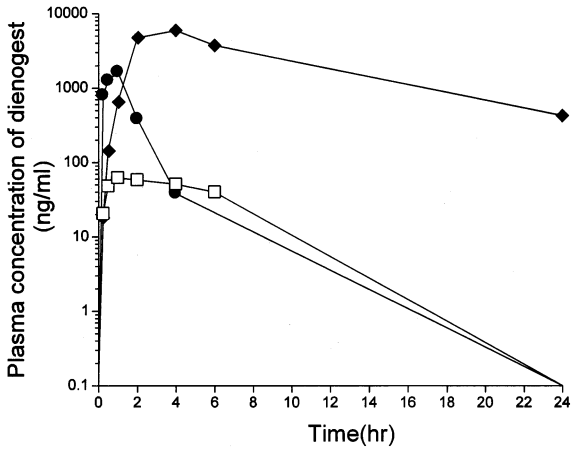

Fig. 6. Plasma concentration-time curves for rats (●), dogs (□), and monkeys (◆) after a single oral administration of dienogest at a dose level of 10, 0.03, or 10 mg/kg, respectively. Each point represents the mean ($n = 5$ for rats, and $n = 3$ for dogs and monkeys).

gland but rather influence proliferation through a prominent secretion of GH from the pituitary gland. However, there are few reports on hypermastia and an increase in lactation in other experimental animals treated with progestogens or progesterone alone. It is recognized that serum levels of GH in women taking oral contraceptives including progesterone and estrogen do not increase (Mishell et al., 1977 and Johnson, 1989).

We determined plasma levels of GH in rats, dogs, and monkeys treated orally with dienogest for 91 days. As a result, rats and monkeys without hypermastia showed no increase in GH level; contrarily, all dogs with mammary hyperplasia showed a prominent increase in plasma GH level in a dose-dependent fashion. These results are in accord with those described by Neumann (1991), Concannon et al. (1981), and Gräf and El Etreby (1979) with other progestins.

In order to examine the direct effect of dienogest on mammary epithelial cells, we conducted an in vitro study using the cells from pregnant rats and lactating dogs as well as human mammary epithelial cells. As a result, dienogest had no direct effect on the growth of mammary epithelial cells from dogs, rodents, or humans at a maximal concentration of 3.2×10^{-8} M in the absence of estradiol. In the presence of estradiol, however, the drug caused the growth of mammary epithelial cells from dogs alone in a concentration-dependent manner. MPA used as a reference progestin showed similar results as obtained with dienogest. These results from the in vitro study also suggest that there is a species difference in the effects of progestational drugs on the mammary gland.

In conclusion, dienogest with progestational activity caused proliferation of mammary gland epithelial cells in dogs through the same physiologic mechanism (increased secretion of GH) as other progestational compounds. This change was specific to dogs. From the results of the in vitro study, the proliferation of canine mammary gland cells may be partially dependent on the direct effect of progestational compounds.

Acknowledgements

We wish to thank Mr. Hiroyuki Murano for his statistical analytical assistance.

Table 2
Pharmacokinetic parameters of dienogest in rats, dogs, and monkeys[a]

Species	Number of animals	Dienogest (mg/kg)	Cmax (ng/ml)	AUC (ng·h/ml)
Rat	5	10	1639 ± 562	2893 ± 732
Dog	3	0.03	62 ± 4	654 ± 50
Monkey	3	10	5942 ± 1914	60411 ± 15106

[a] Values are expressed as the mean $\pm$ SD.

References

Concannon, P.W., Spraker, T.R., Casey, H.W., Hansel, W., 1981. Gross and histopathologic effects of medroxyprogesterone acetate and progesterone on the mammary glands of adult beagle bitches. Fertil. Steril. 36, 373–387.

Gräf, K.J., El Etreby, M.F., 1979. Endocrinology of reproduction in the female beagle dogs and its significance in mammary gland tumorigenesis. Acta Endocrinol. Suppl. 222, 1–34.

Hemmilä, I., 1988. Lanthanides as probes for time-resolved fluorescentric immunoassays. Scand. L. Clin. Lab. Invest. 48, 389–400.

Hoffmann, H., Hillesheim, H.G., Guttner, J., Stade, K., Merbet, E.M., Holle, K., 1983. Long term toxicological studies on the progestin STS 557. Exp. Clin. Endocrinol. 81, 179–196.

Johnson, A.N., 1989. Comparative aspects of contraceptive steroids — effects observed in beagle dogs. Toxicol. Pathol. 17, 389–395.

Katsuki, Y., Sasagawa, S., Takano, Y., Shibutani, Y., Aoki, D., Udagawa, Y., Nozawa, S., 1997. Animal studies on the endocrinological profile of dienogest, a novel synthetic steroid. Drugs Exp. Clin. Res. 23, 45–62.

Katsuki, Y., Takano, Y., Futamura, Y., Shibutani, Y., Aoki, D., Udagawa, Y., Nozawa, S., 1998. Effects of dienogest, a synthetic steroid, on experimental endometriosis in rats. Eur. J. Endocrinol. 138, 216–226.

McCann, J.P., Altszuler, N., Hampshire, J., Concannon, P.W., 1987. Growth hormone, insulin, glucose, cortisol, luteinizing hormone, and diabetes in beagle bitches treated with medroxyprogesterone acetate. Acta Endocrinol. 116, 73–80.

Mishell, D.R., Kletzky, O.A., Brenner, P.F., Roy, S., Nicoloff, F., 1977. The effect of contraceptive steroids on hypothalamic-pituitary function. Am. J. Obstet. Gynecol. 128, 60–74.

Neumann, F., 1991. Early indicators for carcinogenesis in sex-hormone-sensitive organs. Mutat. Res. 248, 341–356.