Regression of Rat Mammary Tumors by a Potent Luteinizing Hormone-releasing Hormone Analogue (Leuprolide) Administered Vaginally

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ABSTRACT

Potent luteinizing hormone-releasing hormone analogues are known to cause regression of hormone-dependent mammary tumors. We have observed that high and long-lasting serum levels of a potent luteinizing hormone-releasing hormone analogue [desglycyl10-(D-leucyl6)] luteinizing hormone-releasing hormone ethylamide, leuprolide] resulted from vaginal administration which effectively caused down regulation in the pituitary by chronic treatment. Regression of 7,12-dimethylbenz(a)-anthracene-induced mammary tumors in Sprague-Dawley rats by consecutive daily vaginal administration of leuprolide was investigated. In untreated rats, 71% of tumors were growing at 8 weeks, whereas after i.p. injection of leuprolide (500 μg/kg) all tumors were regressing 2 weeks after commencement of treatment and 86.7% of tumors disappeared by 8 weeks. Vaginal administration of 100 μg/kg for 8 weeks produced regression in 80% of tumors and disappearance in 35%. The vaginal administration of a higher dose (500 to 5000 μg/kg) produced highly significant antitumor effects [regression in 82.2 ± 4.0% (S.E.) and disappearance in 52.9 ± 2.1%]. These results are consistent with the effects produced by ovariectomy. Whereas 13 and 7 new tumors appeared in untreated rats and those treated vaginally with the effects produced by ovariectomy. The analogue at high doses (7) was administered vaginally once a day for 8 weeks at a dose of 500 μg/kg, respectively, only one or two tumors appeared in i.p. and vaginally (above 500 μg/kg) treated rats during treatment. Histological classification of the mammary tumors after treatment indicated therapeutic effects similar to those shown by tumor size determination. Thus, it was concluded that vaginal application of leuprolide at doses above 500 μg/kg might be a potentially useful method for antitumor therapy.

INTRODUCTION

The potent luteinizing hormone-releasing hormone analogues, desglycyl10-(D-leucyl6) luteinizing hormone-releasing hormone ethylamide (leuprolide) (3, 7, 15, 21) and d-seryl(tertbutyl)6-azoglycyl10 luteinizing hormone-releasing hormone (16), cause regression of hormone-dependent rat mammary tumors. This effect is ascribed to temporary "chemical ovariectomy," gonadal functional atrophy caused by chronic treatment with the analogue at high doses (7). We have observed that vaginal administration of leuprolide to rats results in high and long-lasting serum levels of the analogue, regardless of its rapid disappearance from the blood following i.v. injection (19). Daily vaginal administration of the analogue for 3 to 14 days produced a striking inhibitory effect of gonadotropin-releasing responses of the pituitary, probably as a result of the prolonged stimulation of the target organ (18). Thus, vaginal application of the analogue was proposed as a rational dosage method for self-administra-

RESULTS

Serum levels of leuprolide after i.p. and vaginal administration to diestrous rats are shown in Chart 1. The analogue was rapidly absorbed following i.p. administration and rapidly disappeared from the serum. In cases of vaginal administration, the high and significant antitumor effects [regression in 82.2 ± 4.0% (S.E.) and disappearance in 52.9 ± 2.1%]. These results are consistent with the effects produced by ovariectomy. Whereas 13 and 7 new tumors appeared in untreated rats and those treated vaginally with the effects produced by ovariectomy. The analogue at high doses (7) was administered vaginally once a day for 8 weeks at a dose of 500 μg/kg, respectively, only one or two tumors appeared in i.p. and vaginally (above 500 μg/kg) treated rats during treatment. Histological classification of the mammary tumors after treatment indicated therapeutic effects similar to those shown by tumor size determination. Thus, it was concluded that vaginal application of leuprolide at doses above 500 μg/kg might be a potentially useful method for antitumor therapy.

The analogue was administered i.p. and vaginal administration were determined, in duplicate by the double-antibody radioimmunoassay method (26). The serum samples were stored at −20°C until the radioimmunoassay was performed and were diluted to an adequate concentration with 0.01 M phosphate buffer (pH 7.2) before the assay.

Mammary tumors were induced in the 6-week-old female rats by a single p.o. administration (15 mg/rat/0.5 ml in olive oil) of DMBA (Wako Pure Chemicals, Ind., Ltd., Osaka, Japan). Thirteen weeks after administration, rats bearing tumors were divided into 7 groups, each comprising 5 rats, on the basis of the number (approximately 20/group) and size of the tumors.

The analogue was administered vaginally once a day for 8 weeks at a dose of 100, 500, 1000, 2500, or 5000 μg/kg/400 mg of the jellies. Premature discharge after administration was prevented by the insertion of a cotton ball. The i.p. injection of the analogue as a positive control was conducted once a day at a dose of 500 μg/kg/ml of 0.9% NaCl solution. Tumor size was measured once a week with calipers through the skin, and the mean diameter was calculated as

\[ \frac{\text{Length} + \text{width}}{2} \]

Statistical analysis was carried out by the Wilcoxon rank sum test on the mean diameter. An increase of more than 10% in mean diameter was defined as "growing," a decrease by at least 10% as "regressing," and a change of less than 10% as "static." A tumor that ceased to be palpable was defined as "disappeared." At the end of the 8-week treatment period, all rats were killed, and the tumors recognized initially, ovaries, uterus, and vagina, were removed, fixed in 10% neutral formalin, and stained by hematoxylin and eosin for histological examination.

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2 The abbreviation used is: DMBA, 7,12-dimethylbenz(a)-anthracene.
long-lasting serum levels, 52.9 to 15.5 ng/ml (mean), were observed during 12 hr. The bioavailability over 12 hr assessed by comparison to the serum curve area after i.p. administration was 34.4%.

Typical changes of individual mammary tumor observed in rats treated with leuprolide by i.p. and vaginal routes are shown in Charts 2 and 3. Tumors in nontreated rats grew slowly; the mean diameter increased by approximately 50% in 8 weeks. In rats receiving the analogue (500 µg/kg) i.p., all the tumors were regressing in 1 week and had regressed severely or disappeared by 2 to 4 weeks after the initiation of treatment (Chart 2). Vaginal administration of the analogue (500 µg/kg) also produced regression by 1 week and severe regression 2 to 4 weeks after the start of treatment (Chart 3). The time courses of the regression on responsive tumors were similar at all doses.

Response of rat mammary tumors after i.p. and vaginal administration of leuprolide for 8 weeks and its statistical analysis are shown in Table 1. The tumor size of the i.p. treated rats was drastically decreased (6.2% of initial). A highly significant inhibition of tumor growth as compared to the control animals was induced in the i.p. and vaginally (above 500 µg/kg) treated groups. There was no significant difference (p > 0.1) between any vaginally treated groups; the response in animals treated vaginally with a 100-µg/kg dose of the analogue was significantly different from that in the i.p. treated group (p < 0.02).

The individual tumors were classified by change of the size during the i.p. and vaginal treatment, and time course of the response is shown in Chart 4 as percentage of the initial tumors; a summary of the responses after 8-week treatment is shown in Table 2. Chart 5 shows the number of newly developed mammary tumors. In control rats, the number of growing tumors increased gradually up to 71% after 8 weeks, and 13 new tumors developed. Even in control rats, 29% of tumors spontaneously regressed. Daily i.p. injection of leuprolide markedly suppressed the growth of tumors; all tumors were already regressing 2 weeks after the start of treatment, and 86.7% disappeared in 8 weeks. Only 2 new tumors developed during treatment. Vaginal administration of the analogue (100 µg/kg) also produced regression in 50% of tumors in 2 weeks and in 86.7% in 8 weeks; 35% disappeared. Seven new tumors appeared during treatment. Vaginal treatment with a 500-µg/kg dose of the analogue caused regression of 65.2% of tumors in 2 weeks and 84.2% in 8 weeks; 52.6% disappeared. Much higher doses of the analogue resulted in regression of a magnitude similar to that in the 500-µg/kg treatment group; after the vaginal administration of between 500 µg/kg and 5 mg/kg, regression was observed in 82.2 ± 4.0% (S.E.) of tumors, and 52.9 ± 2.1% were not palpable. Only one or 2 new tumors were observed during these treatments.

Body weight of all treated rats with the analogue progressively increased independently of dose during the treatment.

Most of the 113 tumors recognized initially are classified histologically in Table 3. The growing tumors in control rats were poorly differentiated adenocarcinomas or well-differentiated adenocarcinomas; no changes in the tumor cells were detected in these. Tumors that regressed spontaneously were well-differ-
entiated adenocarcinomas and an adenoma and adenomatous hyperplasia; these showed quite similar changes to those tumors that responded to leuprolide treatment.

In the grossly regressing tumors of the i.p. and vaginal administration groups, flattening of cells, tubule formation, enlargement of tubular lumen of the acini, cells with pyknotic nuclei, and exfoliation of regressed tumor cells were observed (Figs. 1 to 3). In regressing tumors, eosinophilic "rarefaction areas" containing fine fiber-like contents were often observed in the interstitial tissue (Fig. 2). "Disappeared" tumors were macroscopically recognized as a s.c. yellowish nodule which contained tissue consisting of cystic spaces of varying size, lined usually by a single layer of flattened epithelium with pyknotic nuclei (Fig. 3).

Completely regressed tumors in histological observation were defined as tumors with "severe regression". Severe regression was frequently observed by i.p. and vaginal (1 and 5 mg/kg) administration of leuprolide (Table 3). Poorly differentiated adenocarcinomas in all groups were defined as a growing tumor.
administration of all doses of leuprolide were similar (6- to 7-layer vaginal epithelium and stratum corneum with keratohyaline granules, infiltration of neutrophiles, and atrophy of the uterus and ovaries occupied with corpora lutea). In the i.p. injection group, 2- to 3-layer vaginal epithelium without neutrophiles but severe atrophy of the uterus and ovaries was also recognized. Control rats showed change corresponding to stages of the estrous cycle; the morphological appearances of ovaries of a control rat (diestrus) and a leuprolide-treated rat (500 μg/kg vaginally) are shown in Figs. 5 and 6.

**DISCUSSION**

The disappearance of leuprolide from the serum of rats following i.p. administration was rapid, while the vaginal administration in the methylcellulose jelly resulted in the high and long-lasting serum levels and the good bioavailability (34.4% over 12 hr by comparison to i.p. injection data).

Whereas a slow but steady growth of mammary tumors was observed in control rats, treatment with leuprolide by vaginal routes produced rapid regression as seen by i.p. routes; tumor regression was detected 1 to 2 weeks after the start of treatment, and most of the tumors disappeared after 4 to 6 weeks. A 500-μg/kg i.p. injection of the analogue (positive control) produced drastic antitumor activity; all tumors had already regressed 2 weeks after the start of treatment and 86.7% disappeared eventually. Vaginal administration of the analogue also caused highly significant regression of tumors at a dose range of 500 to 5000 μg/kg; 82% of the tumors were regressing, 53% disappeared, and only one or 2 new tumors appeared during treatment (13 tumors were detected in nontreated rats during the same period). Thus, it is clear that the analogue at doses greater than 500 μg/kg not only affects the regression of DMBA-induced rat mammary tumors but also effectively prevents the occurrence of new tumors.

Mammary tumors induced by DMBA are known to be hormone dependent (13, 22, 23, 25); the frequency has been estimated to be 80 to 90% (9, 20). Hence, regression of all DMBA-induced tumors by i.p. administration of leuprolide is an extraordinary result. The regression ratio of tumors by vaginal administration was rapid, while the vaginal administration of all doses of leuprolide were similar (6- to 7-layer vaginal epithelium and stratum corneum with keratohyaline granules, infiltration of neutrophiles, and atrophy of the uterus and ovaries occupied with corpora lutea). In the i.p. injection group, 2- to 3-layer vaginal epithelium without neutrophiles but severe atrophy of the uterus and ovaries was also recognized. Control rats showed change corresponding to stages of the estrous cycle; the morphological appearances of ovaries of a control rat (diestrus) and a leuprolide-treated rat (500 μg/kg vaginally) are shown in Figs. 5 and 6.

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We demonstrated that continuous s.c. and vaginal infusion resulted in an almost thorough down regulation of the gonadotropin responses, which caused endocrine-related atrophy of the ovary, "chemical ovariectomy," and antitumor activity on hormone-dependent tumors. The consecutive daily vaginal administration of the analogue also induced a marked inhibitory effect of gonadotropin-releasing responses at doses of 1 μg/kg or greater due to the long-lasting serum levels of the analogue (18). It is obscure at present why the effective dose on the mammary tumors was about 500 times higher than that on the pituitary down regulation.

The regression effect of leuprolide on the DMBA-induced mammary tumors was estimated by a histological classification as in previous studies (1, 10, 24). Typical histological features of well-regressed tumors were flattening of the cells, vacuolation and enlargement in the acini, pyknotic, and cystic spaces; these observations were consistent with those after ovariectomy, hypophysectomy, and pituitary stalk section (4-6, 11, 12, 27, 28).

**Table 3**

<table>
<thead>
<tr>
<th>Dose μg/kg</th>
<th>No. of examined tumors</th>
<th>Severe regression</th>
<th>Adenoma and adenomatous hyperplasia</th>
<th>Fibroadenoma</th>
<th>Well-differentiated adenocarcinoma</th>
<th>Poorly differentiated adenocarcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>0</td>
<td>1 (1)</td>
<td>0</td>
<td>5 (2)</td>
<td>2 (0)</td>
</tr>
<tr>
<td>I.p.</td>
<td>500</td>
<td>15</td>
<td>13 (13)</td>
<td>0</td>
<td>2 (2)</td>
<td>0</td>
</tr>
<tr>
<td>Vaginal</td>
<td>100</td>
<td>14</td>
<td>0</td>
<td>8 (8)</td>
<td>0</td>
<td>4 (3)</td>
</tr>
<tr>
<td>Vaginal</td>
<td>500</td>
<td>22</td>
<td>4 (4)</td>
<td>6 (6)</td>
<td>1 (0)</td>
<td>10 (9)</td>
</tr>
<tr>
<td>Vaginal</td>
<td>1000</td>
<td>19</td>
<td>7 (7)</td>
<td>2 (2)</td>
<td>2 (1)</td>
<td>8 (8)</td>
</tr>
<tr>
<td>Vaginal</td>
<td>2500</td>
<td>17</td>
<td>3 (3)</td>
<td>3 (3)</td>
<td>2 (2)</td>
<td>7 (6)</td>
</tr>
<tr>
<td>Vaginal</td>
<td>5000</td>
<td>18</td>
<td>10 (10)</td>
<td>3 (3)</td>
<td>0</td>
<td>5 (5)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of tumors with regression.

Although a close correlation between estrogen receptors and the differentiation of tumors has not been proved, the poorly differentiated adenocarcinomas were barely affected by the treatment in the present work. Interestingly, the coexistence of regressing areas and apparently growing areas was observed in one tumor; this may be ascribed to intersite variation of estrogen receptors.

Histological observation of the internal genital organs after i.p. and vaginal administration of leuprolide (Table 3). There was a close correlation between the estimation of the response by the tumor size and histological classification (Table 3).

Although a close correlation between estrogen receptors and the differentiation of tumors has not been proved, the poorly differentiated adenocarcinomas were barely affected by the treatment in the present work. Interestingly, the coexistence of regressing areas and apparently growing areas was observed in one tumor; this may be ascribed to intersite variation of estrogen receptors.

Histological observation of the internal genital organs after treatment showed drastic atrophy of the ovaries (Fig. 6) and supports the suggestion that leuprolide effected the regression of mammary tumors through an endocrine-related atrophy of the ovary (7, 15).

In summary, it was demonstrated by tumor size and histological classification that the vaginal administration of leuprolide at a dose of more than 500 μg/kg could cause the highly significant regression of hormone-dependent rat mammary tumors induced by DMBA and the prevention of new tumors. Thus, the vaginal application of the analogue might be useful as a convenient self-administered method for anti-mammary tumor therapy.

**ACKNOWLEDGMENTS**

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REFERENCES


Fig. 1. Mammary tumor with severe regression after vaginal administration of leuprolide (5 mg/kg) in rat (no. 7-2), showing remarkable increase in tubular lumen with atrophied carcinomatous tissue. H & E, x 57.6.

Fig. 2. Detail of Fig. 1, showing flattening of cells with pyknosis and "rarefaction areas" with eosinophilic fiber-like contents (arrow). H & E, x 576.

Fig. 3. Mammary tumor with severe regression after treatment as in Fig. 1 in rat (No. 7-3), showing extremely flattened cell with pyknotic nuclei and narrowed interstitial tissue. H & E, x 576.

Fig. 4. Well-differentiated adenocarcinoma with regression in rat (No. 7-1) after the vaginal administration of leuprolide (5 mg/kg), showing intersite variation of the effect. Regressing area (above) and nonregressing area (below) in one tumor. H & E, x 57.6.

Fig. 5. Ovary of control rat (No. 1-1), showing an increase in corpora lutea at diestrus. H & E, x 14.4.

Fig. 6. Ovary of rat (No. 4-5) after the vaginal administration of leuprolide (500 μg/kg), showing the striking atrophy of ovary occupied with corpora lutea. H & E, x 14.4.
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