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ABSTRACT

The direct antitumoral effects of gonadotropin-releasing hormone (GnRH) analogues on breast tumors have been surmised from clinical observations and in vitro studies. The present study aimed to determine the effects of the GnRH agonist [d-Trp^6]GnRH (Decapeptyl) on steps of experimental mammary carcinogenesis, and the mechanisms, other than the chemical castration, involved. We chose a recent model, i.e., mammary tumors induced by wild-type A2 polyoma (Py) virus in BALB/c female nu/nu mice, which displays the following characteristics. Tumors are mammary adenocarcinomas similar to well differentiated breast carcinomas. Tumor promotion period ends 20 days after Py virus inoculation and is estradiol dependent. The first palpable tumors occur 60 days after Py virus inoculation, and tumor growth is ovarian hormone independent. The effects of Decapeptyl treatment on tumor induction and tumor growth were studied in normal or ovariectomized 6-week-old nude mice inoculated with 10^3 plaque-forming units Py virus (day 0 of experiments). Normal mice and ovariectomized mice percutaneously supplemented with 0.6 µg 17β-estradiol every other day until day 30 (OvE2 mice) were treated with monthly s.c. injections of the sustained release form of Decapeptyl (5 mg/kg) until the end of 180-day experiments. Overall values for latency periods were included within a day 60 to day 130 time interval. Hormone-independent outgrowth was not affected. We focused on tumor progression before the outgrowth. Incidences on tumor appearance kinetics account for effects at this stage. 17β-Estradiol repletion strongly antagonized (P < 0.001) the slowing effect of ovariectomy on the tumor appearance kinetics, indicating that tumor progression is estradiol sensitive in its early stages. [d-Trp^6]GnRH treatment antagonized tumor appearance profiles, inducing similar kinetics in both normal and OvE2 mice. In normal mice, the antagonism (P < 0.01) was concomitant with significant decreases (P < 0.05) in serum levels of estradiol and prolactin, which are critical hormones for mammary tumor development in mice, suggesting a pituitary-mediated effect. In OvE2 mice, the antagonism (P < 0.01) occurs independently of estradiol and prolactin, suggesting a direct effect at the mammary cell level. Because of alterations in kinetics, this effect is exerted at the early stages of tumor progression on Py virus-transformed, ovarian hormone-sensitive cells in the mammary tissue. This new animal model of breast cancer is shown to be useful in characterizing direct antitumoral effects of GnRH analogues and studying the basic mechanisms of mammary carcinogenesis.

INTRODUCTION

Chronic administration of GnRH agonists at high doses is an effective hormonal therapy for human breast cancer in premenopausal patients (1). The remissions achieved have usually been attributed to suppression of the pituitary-gonadal axis, with a consequent decrease in gonadal steroidogenesis (2). However, an alternative hypothesis involving a non-pituitary-mediated action on tumor is supported by the following observations. Clinical remissions in GnRH agonist-treated postmenopausal breast cancer patients without any endocrine effect (3, 4) have been reported. In vitro studies demonstrate that GnRH inhibits growth of human breast cancer cell lines (5–9). GnRH analogue high and low affinity binding sites have been detected in breast cancer tissues and mouse mammary tumors (10–13). Finally, GnRH analogues act on the phospholipase C-transducing system in mammary tumors (14).

We were interested in investigating the direct antitumoral action of the GnRH agonist [d-Trp^6]GnRH. The best clinical situation for this purpose would be first-line therapy of previously untreated postmenopausal breast cancer with [d-Trp^6]GnRH as a single agent. However, few data are available with respect to such an approach (4, 15). We have shown that [d-Trp^6]GnRH inhibits estrogen-stimulated growth of the human breast cancer cell line MCF-7 (9). The aim of the present study was to evaluate the direct antitumoral effects of this agonist in an animal model of breast cancer.

The model used in this study was chosen for its similarity to human mammary carcinoma. Virus-induced mammary carcinogenesis in the mouse is known to be dependent on genetic and hormonal factors, as in human pathology (16). Wild-type A2 Py virus was recently reported to be highly oncogenic in BALB/c female athymic nude mice, inducing mammary adenocarcinomas histologically very similar to human intraglacto-phoric carcinomas (17, 18). Tumor induction frequency was greatly reduced by ovariectomy and completely restored by administering 17β-estradiol for 20 days after Py virus injection, days 10 to 20 being critical (19). Tumor growth was unaffected by ovariectomy or transplantation from females to males (18). These tumors thus appeared to be 17β-estradiol dependent for their promotion and ovarian hormone independent for growth (18, 19). The altered antitumor immune response in immunodeficient athymic mice is an advantage for the evaluation of antitumoral drug potencies. This animal model thus displays unique features which made it well suited to our investigation.

Since the delay between tumor promotion and tumor outgrowth may be affected by [d-Trp^6]GnRH potency and yet has not been studied in terms of endocrine factor sensitivity, the effects of ovarian hormones on this period were investigated, together with those of [d-Trp^6]GnRH. The experimental approach sought to answer to the following questions. Is ovarian hormone-independent tumor growth inhibited by [d-Trp^6]GnRH directly at the tumor level? Is this GnRH agonist able to directly affect the ovarian hormone-sensitive stages of the mammary tumoral process? What is the relative importance of these effects and those of endocrine status? We postulated that antagonism of carcinogenesis restored by exogenous 17β-estradiol in ovariectomized mice by [d-Trp^6]GnRH would occur without endocrine effects, i.e., as a result of direct antitumoral effects. Since 17β-estradiol and prolactin are critical in pro-
moting mouse mammary carcinogenesis (20), serum levels of these two hormones were monitored during experiments.

MATERIALS AND METHODS

Peptide and Chemicals. [d-Trp6]GnRH (Decapeptyl) microcapsules were provided by IPSEN Biotech (Paris, France). 17β-Estradiol, estrol, estrone, testosterone, and cortisol were obtained from Steraloids Inc. (Wilton, NH). These compounds were diluted in ethanol at appropriate concentrations and stored at -20°C. Mice and Virus. Four-week-old normal or ovariectomized female athymic nude mice (BALB/c nude) were purchased from IFN CREDO (L’Arbresles, France). Animals were kept in an isolator (Flu- France, Cachan, France). Sterilized food and sawdust bedding were obtained from PALA (Marseille, France). Drinking water was autoclaved. Wild-type A2 polyoma virus (21) was provided by Dr. M. Berebbi (IRSC, Villejuif, France).

Experimental Model. The susceptibility of athymic nude mice to mammary tumor induction by the Py virus has been shown to depend on immunological, genetic, and hormonal factors (17). In 6-week-old BALB/c female nude mice, mammary adenocarcinomas appear with a latency period of at least 2 months after Py virus inoculation. Tumor volume reaches about 1 cm³ within the 15 days following appearance. The over-day 20 period after Py virus inoculation is not critical with respect to tumor frequency. Day 20 is thus considered as marking the end of the promotion period and the beginning of tumor progression (18). Unlike estrogen-dependent promotion, for which effects are indicated by the incidence on tumor frequencies, the last stage of tumor progression is ovarian hormone independent, as illustrated by autonomous outgrowth in spite of the fact that tumor cells bear functional estradiol and progesterone receptors (18). In order to study the sensitivity of tumor progression to ovarian hormones in the early phase before outgrowth, we studied the incidence on tumor appearance kinetics. The effects of both ovarian hormones and continuous [n-Trp6]GnRH treatment on mammary tumor development in normal mice and OvE2 mice were investigated as indicated below.

In Vivo Experiments. At the start of the experiments, 6-week-old mice were divided into the following groups: normal mice, normal mice treated with [d-Trp6]GnRH, Ov mice, OvE2 mice, and OvE2 mice treated with [d-Trp6]GnRH. In all groups, wild-type A2 Py virus was inoculated by a single s.c. dorsal injection of 10⁵ PFU, as described (16). OvE2 mice received percutaneously 0.6 μg 17β-estradiol diluted in 30 μl ethanol and were compensated every other day for 30 days after Py virus injection. Ov mice received the vehicle. In the two groups treated with [d-Trp6]GnRH, 200 μl of Decapeptyl microcapsules were injected s.c. into the back 2 days prior to Py virus inoculation. Since it has been reported that in mice the sensitivity of the pituitary-gonadal axis to the inhibitory effects of GnRH agonists is less than in other species (22), a dose of 5 mg/kg [d-Trp6]GnRH (100 μg/mouse) was used. Decapeptyl injections were repeated monthly until the end of the experiments. Untreated groups received 200 μl of vehicle. Two studies were carried out, i.e., a 30-day and a 180-day experiment. The purpose of the 30-day experiment (8 mice/group) was to determine the effects of ovarioectomy, 1-month 17β-estradiol repletion, and 1-month Decapeptyl treatment on estradiol and prolactin serum levels in female athymic nude mice inoculated with Py virus. On day 30, trunk blood was collected and plasma was prepared for hormonal dosages. In the 180-day experiment (8 mice/group, except for control Ov mice, 16 mice), each animal was inspected for tumors once a week for the first month and 3 times a week for the next 5 months. Ten days after the appearance of the first palpable tumor(s), mice were weighed, trunk blood was collected, and plasma was prepared for hormonal dosages. Tumors were carefully removed, weighed, and measured with microcalipers. After taking a piece of tumor tissue for histological examination, the rest of the tumor was quickly frozen in liquid nitrogen pending biochemical analyses. Tumor volume was calculated as described (23):

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Tumor\, volume = length \times width \times height \times 0.5236
\]

Hormonal Assays. Serum estradiol levels were measured using the antiserum and labeled hormone of the radioimmunoassay kit supplied by CIS (Gif-sur-Yvette, France). In order to lower the detection threshold, diethyl ether extraction was performed on 50-μl serum samples, as described (24), with recovery of serum steroids in 50 μl of buffer (0.14 M NaCl, 1.4 mM KH₂PO₄, 16 mM Na₂HPO₄·2 H₂O, 2.7 mM KCl) containing 0.5% bovine serum albumin and 2.5% ethanol. The range of sensitivity was 2–100 pg/ml. Prior to serum assays, we checked that the antiserum displayed low cross-reactivity with other extracted steroids such as estrone, estriol, testosterone, and cortisol (≤0.1% in all cases). Values were corrected for procedural losses (factor of 1.1).

Serum prolactin levels were measured by double-antibody radioimmunoassay, using mouse prolactin radioimmunoassay reagents provided by Dr. A. F. Parlow (Pituitary Hormones and Antisera Center, Torrance, CA). Mouse prolactin was iodinated as previously described (25). Assays were performed on 25-μl serum samples. The range of sensitivity was 1–200 ng/ml.

Statistics. Tumor growth and hormone serum levels were analyzed using a computed ANOVA. Differences between two groups were considered significant if both Fisher’s and Scheffé’s F tests were significant (26, 27). Tumor incidence curves were generated by the Kaplan-Meier method (28) and compared by log rank analysis (29). Kinetics on tumor appearance were represented by linear regressions of data (30). The P value for each regression was assessed by computed ANOVA using Fisher’s test. To assess whether kinetics between two groups were significantly different, regression slopes were compared according to the following formula (30):

\[
t = \left| \frac{\text{slope}_1 - \text{slope}_2}{(1/a_1 + 1/a_2)^{1/2}} \right| (w_1 + w_2)/(n_1 + n_2 - 4)^{1/2}
\]

where n is the (x,y) pair number, a is the square sum on (x - mean, y), and w is the residual square sum. Slopes were assessed differently at the risk P if it was found to be superior or equal to the value given by t tables for the n₁ + n₂ - 4 degree of freedom. A P value of < 0.05 was considered significant in both tests.

RESULTS

Effects of Long Term [d-Trp6]GnRH Treatment on Mammary Tumor Outgrowth. All tumors were classified as mammary adenocarcinomas. This finding is in line with previous reports using wild-type Py virus (17). Treatment with Decapeptyl microcapsules did not affect histological type (not shown). As indicated in Table 1, body weight was similar in all groups. The incidence of tumor induction observed in normal control mice was fully conserved in OvE2 mice treated or not with [d-Trp6]GnRH. Tumor growth was not altered by ovarioectomy and was not significantly affected by long term [d-Trp6]GnRH treatment in normal or OvE2 mice. The ovarian hormone-independent stage of tumor growth was, therefore, insensitive to Decapeptyl action occurring directly at the tumor level.

Endocrine Effects of 1-Month or Long Term Treatment with [d-Trp6]GnRH. Fig. 1 shows the effects of short term (Fig. 1A) or long term (Fig. 1B) treatment with [d-Trp6]GnRH on the serum levels of estradiol and prolactin in normal and OvE2 mice. In normal mice, control serum levels were significantly affected by [d-Trp6]GnRH for long term treatment only (Fig. 1). In OvE2 mice, increases in estradiol and prolactin levels after 1-month estradiol repletion (Fig. 1A) did not persist beyond day 60 (Fig. 1B).

Our main results are the following. Ovariectomy had a significant inhibiting effect on both estradiol and prolactin serum levels (Fig. 1). The period of [d-Trp6]GnRH administration required for the effective suppression of the gonadal estradiol synthesis with decreased prolactin serum levels in normal mice is over 1 month (Fig. 1). One-month estradiol repletion efficiently restores estradiol and prolactin serum levels in OvE2 mice. This finding is in line with previous reports using wild-type Py virus (17). Treatment with Decapeptyl microcapsules did not affect histological type (not shown). As indicated in Table 1, body weight was similar in all groups. The incidence of tumor induction observed in normal control mice was fully conserved in OvE2 mice treated or not with [d-Trp6]GnRH. Tumor growth was not altered by ovarioectomy and was not significantly affected by long term [d-Trp6]GnRH treatment in normal or OvE2 mice. The ovarian hormone-independent stage of tumor growth was, therefore, insensitive to Decapeptyl action occurring directly at the tumor level.

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Effects of Long Term [D-Trp^6]GnRH Treatment on the Tumoral Process. Tumor incidence is expressed as a function of the latency period, i.e., the period of time between Py virus inoculations and palpable outgrowth, in Fig. 2. It is represented two ways. Kaplan-Meier curves (Fig. 2A) are used to represent overall responses of the mice, regardless of group, to the different treatments used. The log rank analysis was used to test the divergence between predicted values and observed values in two compared groups (26). Linear regression was used on data from tumor-bearing mice (Fig. 2B) to focus analysis on tumor appearance kinetics. These two aspects will be described separately and discussed together with results relative to estradiol and prolactin serum levels (Fig. 1).

As shown by Kaplan-Meier curves, all untreated normal mice had palpable mammary tumors within the day 60 to day 80 latency period. As compared to this group, ovariectomy significantly altered the tumor-induction profile, concomitant with decreasing estradiol and prolactin serum levels (Fig. 1B). In ovariectomized mice 1-month estradiol repletion led to restoration of a tumor-induction profile similar to the one obtained in normal mice. In both normal and OvE2 mice, long term administration of Decapeptyl significantly antagonized the mammary tumoral process and led to two similar profiles. Unlike normal mice, where endocrine effects were observed, the antagonism in OvE2 mice occurred without any significant change in estradiol and prolactin serum levels (Fig. 1B), and the tumor induction profile was significantly different from that of untreated Ov mice.

As shown in Fig. 2B, the kinetics of tumor appearance were affected by hormonal manipulations of mice. Changes in this parameter further characterize effects on tumor development. Ovariectomy significantly lowered the regression slope, but the estradiol repletion and subsequent increased prolactin serum levels (Fig. 1A) were not efficient enough to restore normal kinetics. Decapeptyl treatment significantly decreased slope values in both normal and OvE2 mice. Tumor appearance kinetics were identical in these two groups and remained higher than in untreated Ov mice, which were less susceptible to Py virus oncogenicity. These results indicate that the effect of [D-Trp^6]GnRH on appearance kinetics was maximal in both normal and OvE2 mice, i.e., with or without endocrine effects (Fig. 1).

DISCUSSION

Several studies have been carried out on the inhibition by GnRH agonists of the growth of hormone-dependent mammary tumors in rodent models (31–35). However, these experiments were focused mainly on the suppressive effect of these molecules on sex steroid and serum prolactin levels. In the present study, we investigated the direct potency of Decapeptyl on the Py virus-induced tumoral process in the mammary tissue of athymic nude mice. In addition to displaying similarities with human evolutive situations, this model provides insight into the direct effects at the level of mammary cell populations during the tumoral process (17–19). The successive stages occurring during this tumoral process are represented in the schematic overall diagram (Fig. 3), which includes data from both previous studies (18) and the present investigation.

The mammary tumors observed in untreated normal mice were similar to those previously described (18) with regard to histopathological profile and estrogen and progesterone receptor levels (not shown). The frequency parameter for tumor induction was shown to account for effects on the hormone-dependent tumor promotion (18). In this study, we focused on the kinetic parameter, which accounts for effects on the tumor progression.

In normal mice, the endocrine effects of Decapeptyl on estradiol and prolactin levels occur through pituitary desensitization (2) as well as likely through direct inhibition of ovarian steroidogenesis (36, 37). The effectiveness of 5 mg/kg doses in holding down serum estradiol levels in nude mice for treatment periods lasting over 2 months is in agreement with a recent report of Bokser et al. (38), who established that 5-month treatment with 2.5 mg/kg doses of Decapeptyl microcapsules significantly decreased luteinizing hormone and estradiol serum levels in adult female Swiss CD-1 mice. One can consider that antagonism of tumor induction by [D-Trp^6]GnRH results in part from endocrine effects on estradiol, prolactin, and, probably, progesterone (34). Since suppression in our experiments was not noted on day 30 but was from day 60, it is likely that pituitary-mediated inhibition of mammary carcinogenesis in normal mice takes place in this day 30 to day 60 interval and beyond. As described above, the over-day 20 period corresponds to the first steps of tumor progression, when hormonal modulation is illustrated by changes in kinetic parameters. The fact that the kinetics of tumor appearance were affected by the [D-Trp^6]GnRH treatment indicates ovarian hormone sensitivity at the beginning of progression. Although not significant as compared to control, tumor frequency in treated mice suggests that the interactions between Py virus, ovarian hormones, and mammary tissue at the promotion steps were not optimal for the full induction of tumors.
Fig. 1. Endocrine effects of 17ß-estradiol and/or [D-Trp6]GnRH administration on serum estradiol and prolactin levels in female BALB/c athymic nude mice inoculated with wild-type A2 Py virus. Normal or ovariectomized 6-week-old BALB/c athymic nude mice were given s.c. injections of 10^7 PFU Py virus and subjected 2 days later to treatment, as follows. N, normal mice; ND, normal mice treated monthly with a single s.c. injection of 0.6 μg/animal of estradiol every other day until day 30; Ov, ovariectomized mice; OvE2, ovariectomized mice supplemented percutaneously with estradiol (0.6 μg/animal) every other day during the first month of treatment on scrum estradiol and prolactin levels in female BALB/c athymic nude mice inoculated with wild-type A2 Py virus. Normal or ovariectomized 6-week-old BALB/c athymic nude mice were given s.c. injections of 10^7 PFU Py virus and subjected 2 days later (day 0) to treatment, as follows. N, normal mice; Ov, normal mice treated monthly with a single s.c. injection of [D-Trp6]GnRH microcapsules (5 mg/kg); OvE1, ovariectomized mice; OvE2, ovariectomized mice supplemented percutaneously with estradiol (0.6 μg/animal) every other day (0.6 μg/animal) until day 30. OvE2 mice as compared to those of Ov mice is evidence that the conditions for hormone-dependent tumor promotion were satisfied. In addition, restoration of kinetics in OvE2 mice as compared to those of Ov mice is evidence that the first steps of the tumor progression are estradiol sensitive. However, the fact that the restored kinetics are slower than in normal mice indicates that prolonged administration of estradiol and other hormones such as progesterone would probably be required to fully restore tumor appearance kinetic (39).

This occurs without any change in the serum levels of estradiol and prolactin, the only two hormones which could influence the mammary tumoral process (20, 39). These observations strongly suggest that the observed antagonism results from a direct antitumoral effect of Decapeptyl. Since only the kinetics were affected, this direct effect probably occurs during the first steps of tumor progression. Because the profiles of tumor induction in both oV: and normal mice under Decapeptyl treatments were similar, the direct antagonistic effect on tumor progression appears to be as potent as chemical suppression.

The direct action of [D-Trp6]GnRH on tumor progression appears at the ovarian hormone-sensitive early stages. This result is in agreement with the conclusions of our previous in vitro study (9), in which growth inhibition by [D-Trp6]GnRH was observed only on hormone-sensitive human breast cancer cell lines. Our findings are in accordance with those of De Launoit et al. (40), who demonstrated direct inhibition by Gnordorelin of initiated thyroidine incorporation in transplantable MXT hormone-dependent mouse mammary tumors. Taken together, these data suggest that GnRH agonists can directly affect growth factors in hormone-sensitive mammary tumor tissue.

From a clinical standpoint, the findings reported herein suggest that GnRH agonists, which are widely used in cancer therapies for their endocrine effects, might also be used for their direct antitumoral potency. This effect could be useful especially in postmenopausal breast cancer, where direct antitumoral effects of GnRH agonists were thought to play a major role in remission (3, 4). Several studies have provided insight into the clinical importance of GnRH agonist direct antitumoral effects (41-45). Clinical reports on Decapeptyl are scarce (4, 15), and our data strengthen the hypothesis that, thanks to its direct action, this drug would be potent for therapy in postmenopausal breast cancer patients. Such a mechanism is, however, restricted to hormone-sensitive mammary tumor cells, and [D-Trp6]GnRH has no effect on hormone-independent tumors. New GnRH antagonists have recently been shown to inhibit the growth of the hormone-insensitive human breast cancer cell line MDA MB 231 (46). As an in vivo approach, the effects of such GnRH antagonists on the hormone-independent growth of Py virus-induced mammary tumors can be studied and we are currently conducting such an investigation.

The main findings of this report can be summed up as follows. Evidence is provided for the ovarian hormone sensitivity of the early mammary tumor progression in BALB/c athymic mice inoculated with Py virus. [D-Trp6]GnRH is shown to have a potent direct inhibiting effect on this stage of mammary tumor development. This study also confirms the usefulness of this experimental model in investigating the direct antitumoral action of GnRH analogues on the mammary tissue and in studying the basic mechanisms of mouse mammary carcinogenesis.

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Direct Inhibiting Effects of [d-Trp⁶]Gonadotropin-releasing Hormone on the Estrogen-sensitive Progression of Polyoma Virus-induced Mammary Tumors in Athymic Mice

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