Rapid Induction of Ovarian Granulosa Cell Tumors by 7,12-Dimethylbenz(a)anthracene in Neonatally Estrogenized Mice

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ABSTRACT

Groups of female C3H/He Ms x 129/J F1 mice were given injections of either 20 μg of 17β-estradiol or sesame oil (vehicle) for the first 5 days after birth. Half of each group was then given gastric intubations of 20 mg/kg of 7,12-dimethylbenz(a)anthracene (DMBA) at 70, 77, and 84 days of age. The other half of each group was given sesame oil. Thus, this design yielded four experimental groups: oil + oil; 17β-estradiol + oil; oil + DMBA; and 17β-estradiol + DMBA. They were sacrificed at approximately 144 days of age (Experiment 1) or the day of palpable ovarian tumor detection or 360 days of age (Experiment 2). In Experiment 1, the total number of oocytes (follicles) per ovary in mice of the 17β-estradiol + oil group was maintained at the same level as mice of the oil + oil group. A significant reduction of oocytes, however, was observed in mice of the 17β-estradiol + DMBA group in comparison with mice of the oil + DMBA group (P < 0.01), and neoplastic nodules of the granulosa cell type developed in the unilateral ovary in 10 of 17 mice of the 17β-estradiol + DMBA group. No tumors were detected in mice of the other groups. These results strongly indicate that an abnormal endocrine milieu caused by neonatal treatment with estrogen may induce a high frequency of transformation of some ovarian tissues and rapid growth of the ovarian tumors after DMBA treatment.

INTRODUCTION

It is now well known that DMBA is by far the most potent chemical carcinogen to induce ovarian tumors of the granulosa cell type which are of a low or moderate grade malignancy in rodents (1–6). Ovarian tumors in animals can also develop from treatment with: X-irradiation (7, 8); intrasplenic grafting of ovarian tissues to castrated animals (9); or neonatal thymectomy (10, 11). Furthermore, a high incidence of spontaneous ovarian tumor development is observed in certain strains of mice (12, 13). In each case of ovarian tumorigenesis, the rapid disappearance of oocytes plays an important role in the subsequent development of ovarian tumors, because normal follicles are seldom seen after the appearance of tumor nodules in the ovaries (6, 8, 14). We are convinced that overstimulation of the ovaries with gonadotropic hormones is an indispensable condition for ovarian tumorigenesis. This contention is supported by higher levels of gonadotropic hormones before the appearance of the ovarian tumors in mice with mutant alleles of the W-series (15) and in neonatally thyromectomized mice (16).

It is believed that neonatal treatment of female rodents with estrogen induces a permanent pattern of tonic release of the gonadotropins resulting in the persistent secretion of estrogen from the ovaries in adulthood, since the vaginal smears of these animals show persistent estrus (17). Previously Nagasawa et al. (18) demonstrated with radioimmunoassay techniques that the pituitary of neonatally estrogenized adult female rats constantly secretes considerable amounts of gonadotropic hormones. In the present study with mice, we found that neonatal treatment with estrogen followed by adult treatment with DMBA induces rapid ovarian tumorigenesis.

MATERIALS AND METHODS

Animals. C3.129 mice were used in this experiment. The mice were raised at the Aichi Cancer Center Research Institute in an environmentally controlled room (14-h light from 6 a.m. to 8 p.m.).

Experimental Procedures. Pregnant female C3H/HeMs mice that had been mated with male 129/J mice were put into separate cages to give birth. Sixty-three female pups were given five daily s.c. injections of 20 μg of 17β-estradiol (Sigma Chemical Co.) dissolved in 0.02 ml of sesame oil, starting within approximately 24 h after birth. As a control, 57 female pups were given five daily injections of 0.02 ml of sesame oil. About half of each group was then given three weekly p.o. doses of 20 mg/kg of DMBA (Eastman Organic Chemicals) dissolved in 0.1 ml of sesame oil by a stomach tube, starting 70 days after birth. As a control, the remaining mice were given sesame oil in the same way. Thus, the experiment consisted of 4 groups of mice: oil + oil; 17β-estradiol + oil; oil + DMBA; and 17β-estradiol + DMBA. The animals were sacrificed at either approximately 144 days (Experiment 1) or 360 days (Experiment 2) of age. The animals in Experiment 1 were monitored for their estrous cycle pattern by daily examination of vaginal smears. The oil + oil- and oil + DMBA-treated mice were killed on the day of diestrus between 142 and 147 days of age. The vaginal smear records of the 17β-estradiol + oil- and 17β-estradiol + DMBA-treated mice showed continuous estrus; therefore, both of these groups of mice were killed at 144 days of age. The mice in Experiment 1 were killed between 10 and 11 a.m. by decapitation over heparinized funnels. The plasma recovered after centrifugation was stored at −80°C until assayed. In Experiment 2, some mice were killed before 360 days of age if there was palpable evidence of an ovarian tumor. Ovaries were macroscopically examined, incised if the ovaries had fluid cysts, weighed, fixed in Bouin's solution, and embedded in paraffin for histological examination. The ovaries from each mouse of Experiment 1 were serially cut into sections at 6 μm and then stained with hematoxylin and eosin. The oocytes at the level of the nucleus were counted in every sixth section of each right ovary from mice of the oil + oil, 17β-estradiol + oil, and oil + DMBA groups, and from both ovaries from mice of the 17β-estradiol + DMBA group. Double the number of counting records were used to estimate the total number of oocytes per ovary. The statistical significance of oocyte numbers was determined by the Student t test. Pituitary, thyroid, lungs, liver, stomach, pancreas, spleen, lymph nodes, adrenals, mammary glands, kidneys, salivary glands, uterus, and vagina were also examined histologically. The statistical significance of ovarian tumor incidence was checked by the x² test.
Hormone Assays. All sera obtained from the mice in Experiment 1 were assayed for FSH and LH as previously described (16). Values for the two assays were expressed in terms of the respective NIAMDD-RP-1 reference per ml of plasma. The statistical significance of hormone levels was determined by the Student t test.

RESULTS

Experiment 1. The ovarian tumor incidence of Experiment 1 (animals killed at approximately 144 days of age) is shown in Table 1. In all mice of the oil + oil group, both ovaries showed normal histology (Fig. 1). In all mice of the 17β-estradiol + oil group, both ovaries had various stages of developing follicles but no corpora lutea, and the interstitial cells of the ovaries were composed of light-staining cells (Fig. 2). The total number of oocytes (follicles) per ovary of this group was at much the same level as that of the oil + oil group (Table 2). In all mice of the oil + DMBA group, no remarkable abnormalities were found; however, the number of follicles was significantly reduced in comparison with those of the oil + oil and 17β-estradiol + oil groups, while the number of corpora lutea was increased (Fig. 3). In some mice of this group, luteinized stromal cells were observed in ovaries. Oocyte loss due to DMBA treatments significantly progressed in the neonatally estrogenized mice in comparison with the vehicle-treated mice (Table 2). In 10 of 17 mice (58.8%) of the 17β-estradiol + DMBA group, the granulosa cell neoplasm was microscopically visible in the unilateral ovary (Fig. 4). Of particular interest was the fact that all of the ovaries with neoplastic nodules still had many small apparently intact follicles (Table 2). The other unilateral ovary of the mice showed atrophic features and had a considerable number of small follicles. The mice of this group without neoplastic signs in their ovaries had ovaries similar to the histology of mice of the 17β-estradiol + oil group, although the number of follicles was significantly reduced.

Plasma levels of FSH and LH in the mice of Experiment 1 are shown in Fig. 5. The levels of both gonadotropins were significantly higher in mice of the 17β-estradiol + oil group than in the mice of oil + oil group. Increased levels of both hormones were found in mice of the oil + DMBA group when compared to those of the mice of oil + oil group. The highest levels of both gonadotropins were detected in the 17β-estradiol + DMBA mice without ovarian tumors. In mice of this group with the ovarian tumor nodule, the levels of gonadotropins were significantly lower than that of the 17β-estradiol + oil group.

Experiment 2. The sacrificial day of this experiment was designed for 12 mo of age, that is, 290 days after the first
treatment with DMBA or sesame oil. The ovarian tumor incidence of Experiment 2 is also shown in Table 1. In all mice of the oil + oil group, both ovaries showed normal histology. In the group given 17β-estradiol + oil, the histological features of the ovaries were similar to the ovaries of the younger mice of the same group, although the number of follicles was slightly reduced. In the group given oil + DMBA, 5 of 15 mice (33.3%) had granulosa cell tumors in a unilateral ovary. The ovary on the other side of the ovarian tumor-bearing mice and both ovaries of the mice in the rest of the group showed atrophic histological features and an absence of any follicular apparatus. In the group given 17β-estradiol + DMBA, 11 mice were killed before the designated termination day because a tumor-like mass in the abdomen was detected by digital examination. All these mice had an ovarian tumor on the unilateral side. Eventually 14 of 18 mice (77.8%) had a granulosa cell tumor in a unilateral ovary. The granulosa cell tumors which developed in both the 17β-estradiol + DMBA and oil + DMBA groups showed similar histology. The weight of ovaries with tumor mass and the day of detection of the ovarian tumors in both the 17β-estradiol + DMBA and oil + DMBA groups are shown in Fig. 6. Early development and rapid growth of the ovarian tumors with a high incidence were found in the 17β-estradiol + DMBA group.

Other tumors were detected in the oil + DMBA mice; 2 (13.3%) had transitional cell tumors in the forestomach, and 1 (6.7%) had leukemia. On the other hand, mice of the 17β-estradiol + DMBA group had 1 (5.6%) transitional cell tumor in the forestomach, 3 (16.7%) adenoacanthomas in the mammary glands, 3 (16.7%) epidermoid tumors in the vagina, 3 (16.7%) leukemias, and 1 (5.6%) endometrial tumor. No metastatic signs of ovarian tumors were found in the mice of these groups. No tumors were observed in the mice of other groups.

**DISCUSSION**

The present study demonstrates that a high incidence of ovarian granulosa cell tumors can be induced in neonatally estrogenized C3.129 mice after adult treatment with DMBA. The latency period of the ovarian tumor development in mice...
given 17β-estradiol + DMBA was considerably shorter than in the mice given oil + DMBA. A similar type of promotion effect with estrogen has been reported by Boylan and Calhoon (19) using the system of rat mammary tumorigenesis. That is, the combination of prenatal exposure to diethilstilbestrol and postnatal treatment with DMBA resulted in a significant increase in the number of palpable mammary tumors per rat as compared to rats treated with DMBA alone.

Up to now, the action of DMBA for ovarian tumorigenesis has been a disputed point. Two theories have been proposed for ovarian tumorigenesis by DMBA administration. (a) A two-phase theory has been proposed by Marchant (20): the first phase is an initiation stage of a neoplastic phase involving destruction of oocytes followed by atresia of the follicular apparatus; the second phase is a promotion stage due to overstimulation of the neoplastic ovarian tissues by gonadotropins. In regard to the first phase, Jull (21) proposed that the effect of DMBA in the induction of ovarian tumor is immediate and direct. The basis for this opinion is that, if ovaries are transplanted in ovariectomized mice from donors treated 24 h previously with DMBA, ovarian tumors of the granulosa cell type develop in a significant number of the recipients. This theory is also supported by the work of Shisa and Nishizuka (5). (b) On the other hand, Krarup (22) made a detailed histological analysis of in situ ovarian tumorigenesis induced by DMBA in mice and concluded that the ovarian tumor development is secondary to the elimination of the oocytes and is, therefore, not caused by DMBA itself. This theory is supported by Hilschfrich (23). Further evidence to support this contention can be found in studies of other mechanisms of ovarian tumorigenesis: genetic depletion of oocytes (12, 15); X-irradiation (8); intrasplenic transplantation of ovary to castrated animals (24); and neonatal thyectomy (10, 16). In these systems, disappearance of almost all oocytes and the follicular apparatus was always observed consistently before ovarian tumor development, and the tubular adenaoma type dominantly appeared prior to actual development of the granulosa cell tumor type. In the present study, the second theory may be the mechanism for tumorigenesis of the granulosa cell type in the mice of the oil + DMBA group. In the mice of the 17β-estradiol + DMBA group, however, the earlier appearance of the granulosa cell tumor nodules in the ovaries having a considerable number of intact follicles strongly suggests that the ovaries are very susceptible to a tumorigenic response to DMBA. Our finding strongly suggests that DMBA acts directly on some ovarian tissue as a tumor initiator.

In the present study, all ovarian tumors developed unilaterally with atrophic ovaries on the opposite side. The unilateral development of ovarian tumors has also been reported by previous investigators (1, 4, 5). It appears likely that the presence of an ovarian tumor on one side inhibits the subsequent development of a tumor in the other side. The ovarian tumorigenesis in the 17β-estradiol + DMBA mice of Experiment 1 suggests that ovarian hormones may be produced immediately after the appearance of a neoplastic nodule. In the ovaries of mice and rats that received treatment of estrogenic hormones at prenatal (25) or neonatal (26, 27) ages, several structural and functional changes such as an enlargement of the interstitial compartment with a clear cytoplasm, absence of corpora lutea, and abnormal steroids synthesis have been reported. Similar histological changes were also observed in the neonatally 17β-estradiol-treated mice of the study. These changes appear to be largely due to an abnormal endocrine milieu caused by estrogen treatment. The structural and functional changes of the ovaries that had been induced by estrogen exposure were recovered when the ovaries were transplanted into ovariectomized control females (26), while intact ovaries transplanted into estrogen-treated females had similar changes that were found in estrogen-exposed ovaries (27). In the present study, the total number of oocytes per ovary in the neonatally 17β-estradiol-treated mice was maintained to a level of the vehicle-treated mice; however, a significant loss of oocytes was found in ovaries of the estrogenized mice after DMBA treatment in comparison with ovaries of the vehicle and then DMBA-treated mice. It is generally accepted that activation of ovarian aryl hydrocarbon hydroxylase by polycyclic aromatic hydrocarbons, such as DMBA and 3-methylcholanthrene, is of great importance in the process of oocyte loss (28) and ovarian carcinogenesis (29, 30). Recently Bengtsson and Rydstrom (31) reported that the activity of DMBA hydroxylase in the rat ovary is obviously increased by the stimulation of gonadotropins. High and persistent levels of gonadotropins in the neonatally estrogenized rat were assayed by Nagasawa et al. (18). In the present study, we also found high levels of gonadotropins in the sera from neonatally estrogenized mice. Activity of DMBA hydroxylase may be increasing in the ovary of the neonatally estrogenized mice with a high level of gonadotropins, and the abnormal endocrine milieu may induce significant loss of oocytes, transformation of some ovarian tissues, and rapid growth of the ovarian tumors after DMBA treatment.

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REFERENCES


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