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Ovary-intact, but not ovariectomized female ACI rats treated with 17β-estradiol rapidly develop mammary carcinoma

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We have examined the ability of 17β-estradiol (E2) to induce development of mammary cancers in the female ACI rat. Continuous treatment with E2, delivered through release from s.c. Silastic tubing implants containing 27.5 mg crystalline hormone, resulted in rapid development of palpable mammary tumors in ovary-intact ACI rats. In a population of 21 E2-treated rats, palpable tumors were first observed following 99 days treatment and 100% of the treated population developed tumors within 197 days. The median and mean times to appearance of first palpable tumor were 143 and 145 days respectively. All mammary tumors were classified as carcinomas and invasive features were observed. Circulating E2 levels in the treated animals at the time of sacrifice averaged 185 pg/ml serum. Mammary tumors were not observed in ovary-intact female ACI rats that were not treated with E2. This is the first report indicating that this naturally occurring estrogen is capable of inducing mammary cancers in the ACI rat strain. Mammary carcinoma did not develop in a population of 11 ovariectomized female ACI rats treated with E2 for a period of 140 days. Circulating E2 levels in the treated ovariectomized animals averaged 207 pg/ml. These data indicate that the ovary modulates estrogen-mediated mammary carcinogenesis in this rat strain. Both ovary-intact and ovariectomized female ACI rats displayed similar susceptibilities to E2-induced pituitary tumors and hyperprolactinemia. Pituitary weight was increased 6.0-fold in ovary-intact ACI rats and 5.3-fold in ovariectomized female rats. Circulating prolactin levels averaged 2318 ng/ml in E2-treated, ovary-intact rats and 2285 ng/ml in E2-treated, ovariectomized ACI rats. These data indicate that estrogen-induced hyperprolactinemia is not the sole factor leading to development of mammary cancers in the E2-treated ACI rat.

Introduction

Numerous epidemiological studies indicate the importance of estrogens in the etiology of human breast cancer (1,2). Both early onset of menarche and late onset of menopause are associated with increased risk of developing breast cancer. It is generally believed that this increased risk results from prolonged exposure of the mammary tissues to the estrogens produced by the ovaries throughout the extended period of reproductive viability. Supporting this assertion are data indicating that bilateral ovariectomy prior to menopause significantly reduces breast cancer risk (3). The anti-estrogen tamoxifen is widely used in the treatment of breast cancer and is presently being evaluated clinically as a preventive agent in women at high risk of developing the disease (4–6). The molecular mechanisms through which estrogens contribute to development of breast cancers in humans are not presently defined.

The ACI rat strain is an inbred line derived from a cross between the August and Copenhagen strains (7). The ACI strain differs from nearly all other inbred rat strains in that ACI rats develop mammary cancers at high incidence when treated with the synthetic estrogen diethylstilbestrol (DES*). The propensity of the ACI rat to develop mammary carcinoma in response to DES treatment was first noted by Dunning *et al.* in 1947 (8) and has been confirmed in several studies, not all of which are cited herein (9–19). Whereas ACI rats develop mammary cancers when chronically treated with certain estrogens, spontaneous mammary cancers are rare in this strain (8,14–16,20,21). Similarly, ACI rats develop relatively few mammary cancers when treated with chemical carcinogens, such as dimethylbenz[*a*]anthracene (DMBA), *N*-methylnitrosourea (MNU) (22,23) or ionizing radiation (13,16). Because of the potential importance of the ACI rat strain as an animal model for examining the role of estrogens in the etiology of human breast cancers, we have examined the ability of the naturally occurring estrogen 17β-estradiol (E2) to induce development of mammary cancers in ovary-intact and ovariectomized ACI rats. Our data indicate that E2 rapidly induces development of mammary carcinomas that often display invasive characteristics and that the ovary modulates mammary carcinoma development in animals chronically treated with E2.

Materials and methods

Treatment of animals

Female ACI rats, either ovary-intact or ovariectomized, were obtained from Harlan Sprague–Dawley (Indianapolis, IN). These animals were housed in a barrier animal facility under controlled temperature, humidity and lighting conditions. This facility is accredited by the American Association for Accreditation of Laboratory Animal Care and is operated in accordance with the standards outlined in *Guide for the Care and Use of Laboratory Animals* (DHHS publication 85-23). All procedures involving live animals were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Ovary-intact animals were allowed continuous access to a standard laboratory chow diet (Harlan Teklad, Madison, WI). Treatment of intact females (n = 21) with E2 was initiated when the animals were 61–63 days of age. A population of three untreated ovary-intact females served as controls. Numerous published studies indicate that ovary-intact ACI rats very rarely develop mammary cancers spontaneously (8,14–16,20,21). In a second experiment, female rats were ovariectomized at 26 days of age and obtained from the vendor at 32 days of age. Upon arrival at our facility, these animals were fed a semi-purified diet formulated in accordance with guidelines of the American Institute of Nutrition (24). E2 treatment of the ovariectomized animals (n = 11) was initiated when the animals reached 45 days of age. A population of untreated (n = 11) ovariectomized females served as controls. All animals were examined weekly

***Abbreviations:** DES, diethylstilbestrol; DMBA, dimethylbenz[*a*]anthracene; MNU, *N*-methylnitrosourea; E2, 17β-estradiol; PRL, prolactin; RIA, radioimmunoassay.

for the presence of palpable mammary cancers for the first 10 weeks following initiation of E2 treatment. Thereafter the animals were examined twice weekly.

Preparation and insertion of E2-containing implants

Silastic medical tubing (0.078 inch inner diameter, 0.125 inch outer diameter; Dow Corning, Midland, MI) was cut into 3 cm lengths. One end was sealed with Silastic Medical Adhesive (Silicone Type A; Dow Corning). After the adhesive had cured, 27.5 mg crystalline E2 (Sigma, St Louis, MO) were loaded into the implant and the open end was sealed with Silastic Medical Adhesive. Implants were allowed to cure for at least 24 h prior to surgical insertion. The back of each animal was shaved and disinfected, the skin was opened with sterile scissors, a s.c. cavity was created over the scapulae using the blunt end of the scissors, an implant was inserted into the cavity and the wound was closed with a sterile 9 mm clip. In the experiments in which ovary-intact animals were examined, the untreated control animals did not receive an implant. In the experiment in which ovariectomized animals were examined, an empty implant was inserted into each of the untreated control animals. The wound clip was removed 14 days following surgery.

Collection and processing of tissues

The animals were killed by decapitation. Trunk blood was collected, allowed to clot at 4°C and centrifuged. The serum was collected and stored at –80°C. Pituitary glands were removed, weighed, fixed in 10% neutral buffered formalin and processed for embedding in paraffin. The location and size of each mammary tumor was noted. Mammary tissues, both tumor and normal, were removed, placed in Lillie's fixative for 24 h and processed for embedding in paraffin. Sections of each mammary tissue specimen were prepared, stained with hematoxylin/eosin and examined by bright field microscopy.

Radioimmunoassay of prolactin and 17β-estradiol

Prolactin (PRL) in trunk blood sera was quantified by double antibody radioimmunoassay (RIA) using an anti-rat PRL monoclonal antibody (Amersham) and methods outlined by the supplier. The sera were diluted where appropriate. PRL standards (Amersham) were calibrated by the supplier against the NIH reference RP2 PRL standards. All samples were assayed in duplicate.

E2 in unextracted trunk blood sera was measured using a coated tube RIA (Diagnostic Products Corp., Los Angeles, CA). Prior to assay, the rat sera were diluted 1:2 in zero calibrator buffer obtained from the vendor. Standards provided by the vendor were similarly diluted. The lower limit of sensitivity of the assay, as performed in our laboratory on diluted rat sera, was 50 pg/ml.

Statistical analysis of data

Differences between means were evaluated using a two-tailed Student's *t*-test. P values ≤ 0.05 were considered statistically significant.

Results

Treatment of ovary-intact ACI rats with E2 resulted in rapid development of mammary tumors. A palpable mammary tumor (~2 mm in diameter) was first detected 99 days after initiation of E2 treatment (Figure 1). The median latency ($n = 21$) to appearance of the first palpable tumor was 143 days and 100% of the treated population displayed palpable tumors within 197 days of initiation of E2 treatment. The mean latency was 145 ± 26 days ($n = 20$ animals). A single E2-treated animal became moribund, apparently due to a pituitary tumor, following 168 days treatment. Although no palpable mammary tumor was detected at the time this animal was killed, microscopic intraductal carcinoma was revealed upon histological examination of mammary tissues from this animal (data not shown). Each animal was killed when its largest mammary tumor reached a size of 1.0–1.5 cm in diameter. Upon necropsy all but one of the E2-treated animals were determined to harbor multiple macroscopic mammary tumors (mean 5.6, range 1–18 tumors/animal). These tumors appeared to be randomly distributed throughout the six pairs of mammary glands. No mammary tumors were observed in a population of three untreated, ovary-intact ACI rats (Figure 1). One of the untreated rats was killed following 20 weeks E2 treatment, so that mammary gland histology could be assessed at a time corresponding to the time point at which 50% of the E2-treated population developed mammary cancers. A second

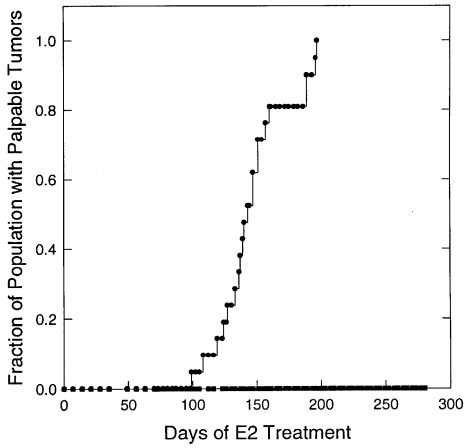

Fig. 1. 17β-Estradiol rapidly induces mammary cancers in female ACI rats. Treatment of ovary-intact female ACI rats with E2 was initiated when the animals were 61–63 days of age. Thereafter the animals were regularly examined for the presence of palpable mammary tumors. A single E2-treated animal with no palpable mammary tumor became moribund, apparently due to a pituitary tumor, following 168 days treatment. Each data point represents the time after initiation of E2 treatment at which an animal exhibited its initial palpable mammary tumor. Filled circle, E2-treated animals; filled square, untreated control animals.

untreated animal was killed at 49 weeks of age. The third untreated rat remained tumor free at the age of 65 weeks; this animal has yet to be killed. Numerous published studies indicate that ovary-intact ACI rats very rarely develop mammary cancers spontaneously (8,14–16,20,21).

All excised tumors were sectioned and histologically classified. The majority of the tumors showed features of intraductal carcinoma of the comedo type. Some of the carcinomas had areas of invasion with a desmoplastic reaction (Figure 2A and B). Some papillary carcinomas were also observed. Mitotic figures were often frequent (Figure 2B). The surrounding mammary tissues displayed marked ductal and lobuloalveolar hyperplasia, often with secretory changes (Figure 2C and D). Microscopic foci of intraductal carcinoma were often observed within these grossly normal mammary tissues. Mammary tissues from untreated, ovary-intact ACI rats were poorly developed, relative to those from E2-treated animals, with virtually no lobuloalveolar tissue identified (Figure 2E and F). Mitotic figures were virtually absent in the mammary tissues from untreated rats.

Pituitary tumors were observed in 100% of the E2-treated, ovary-intact animals. The average length of E2 treatment in this population of animals was 174 ± 28 days. Average pituitary wet weight was increased 6-fold ($P \leq 0.05$) relative to that observed in untreated, ovary-intact animals (Figure 3A). These pituitary tumors produced and released large amounts of PRL, as evidenced by a 42-fold ($P \leq 0.05$) increase in mean circulating PRL in the E2-treated population relative to that observed in untreated animals (Figure 3B). Both pituitary weight and the level of circulating PRL in untreated animals were within normal limits for ovary-intact female rats.

The amount of E2 in trunk blood sera from untreated, ovary-intact ACI rats was <50 pg/ml, the level of sensitivity of the RIA as performed in this study. The mean level of E2 in trunk blood sera from E2-treated, ovary-intact animals averaged 185 pg/ml (Figure 4). During the rat estrous cycle, circulating E2 levels oscillate between ~20 and 75 pg/ml, with peak levels occurring at midday on proestrus (25). The rats in the present

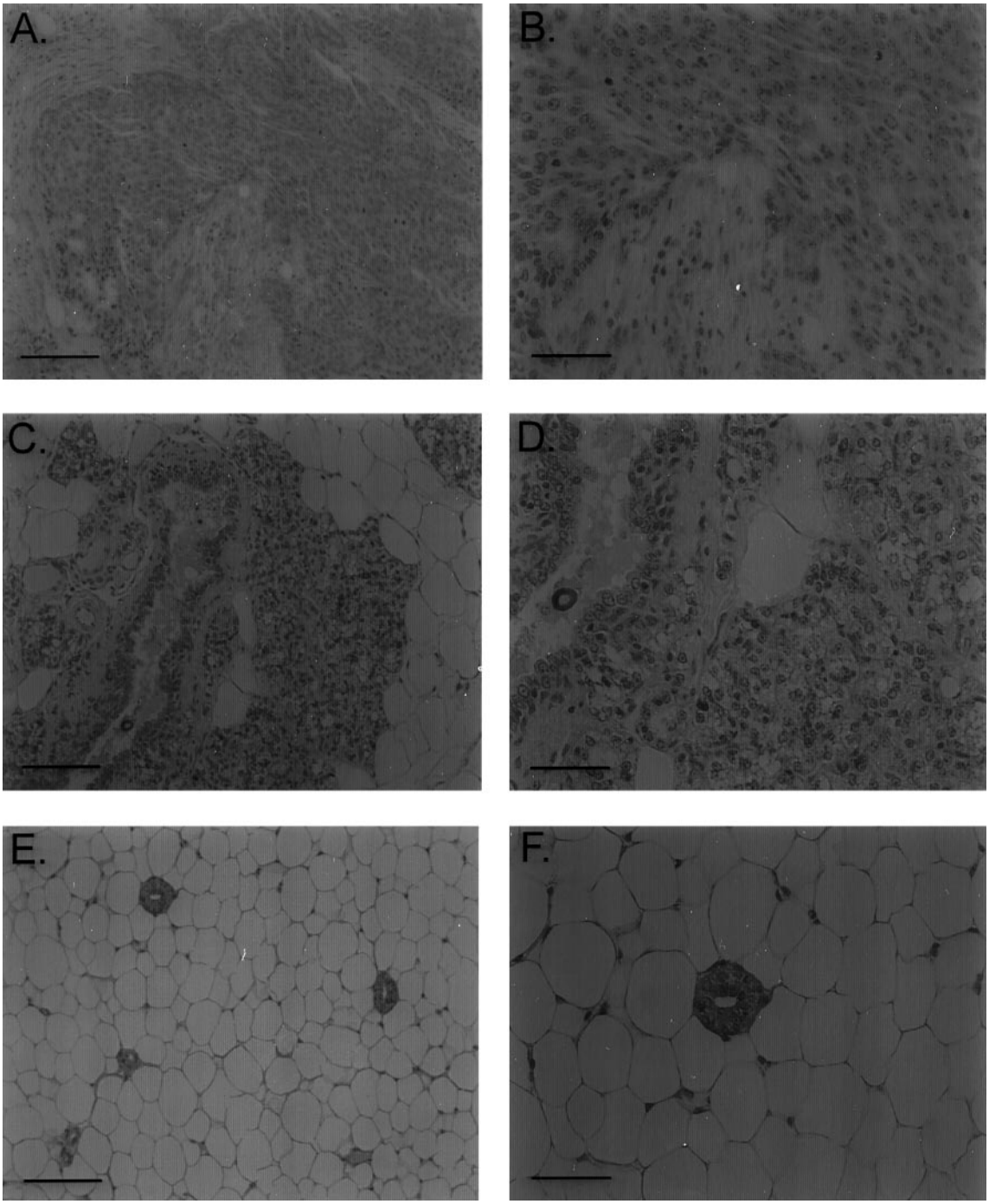

Fig. 2. Histological appearance of mammary tissues from ovary-intact female ACI rats. Each E2-treated animal was killed when its largest tumor was 1.0–1.5 cm in diameter. All mammary tumors and randomly selected regions of grossly normal mammary tissues were processed for histological examination as described in Materials and methods. Representative neoplastic, hyperplastic and normal tissues are illustrated. (**A** and **B**) Mammary carcinoma from a 29-week-old, ovary-intact female ACI rat treated with E2 for 20 weeks. The tumor is invasive and there is a desmoplastic reaction present. (**C** and **D**) Grossly normal, but histologically hyperplastic, mammary tissue from a 29-week-old, ovary-intact female ACI rat treated with E2 for 20 weeks (same animal as in A and B). (**E** and **F**) Mammary tissue from a 29-week-old, ovary-intact female ACI rat not treated with E2. Note the complete absence of developed lobuloalveolar structures. (A), (C) and (E), line indicates 10 μm; (B), (D) and (F), line indicates 5 μm.

study were not evaluated for reproductive cycle. In another experiment in which ovary-intact female rats received E2-containing Silastic implants identical to those used in the present study, the rats exhibited vaginal cytological features indicative of persistent proestrus.

Ovariectomized ACI rats displayed reduced susceptibility to E2-induced mammary carcinoma relative to ovary-intact females. No palpable mammary tumors were detected in a population of 11 ovariectomized females treated with E2 for 140 days (data not shown), an observation in stark contrast to that observed in ovary-intact animals (Figure 1). It is noted that the original purpose of this experiment was to assess, at a defined time point, the ability of administered E2 to promote development of PRL-producing pituitary tumors. Histological examination of grossly normal mammary tissues from these E2-treated, ovariectomized ACI rats revealed ductal and lobulo-

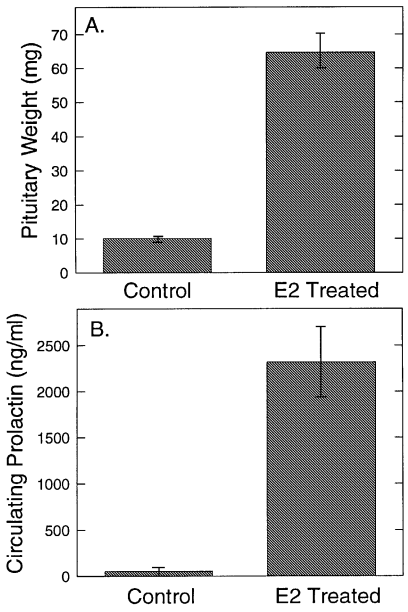

Fig. 3. 17β-Estradiol induces pituitary tumors and hyperprolactinemia in ovary-intact female ACI rats. Animals were treated as described in Figures 1 and 2 and Materials and methods. The average length of E2 treatment was 174 days. Each data bar represents the mean ± SEM (E2-treated, $n = 21$) or range (untreated, $n = 2$). (**A**) Upon sacrifice, the anterior pituitary glands were removed and weighed. (**B**) Trunk blood sera were collected and assayed for PRL by RIA.

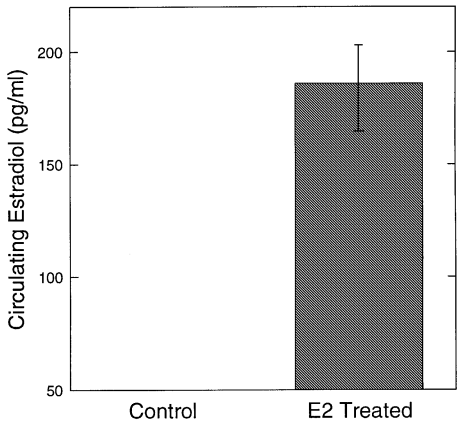

Fig. 4. Level of 17β-estradiol in trunk blood sera from ovary-intact female ACI rats. Trunk blood sera from animals described in Figures 1–3 were assayed for E2 by RIA. E2 levels ⩽50 pg/ml could not be accurately measured. Each data bar represents the mean ± SEM (E2-treated) or range (untreated).

alveolar hyperplasia (Figure 5A and B). However, neither intraductal carcinoma nor infiltrating ductal carcinoma were observed histologically. Mammary tissues from untreated, ovariectomized animals displayed markedly atrophic lobulo-alveolar structures (Figure 5C and D).

Ovariectomized ACI rats treated with E2 for 140 days developed pituitary tumors at an incidence of 100%. Average pituitary wet weight was increased 5.3-fold ($P \leqslant 0.05$) relative to that observed in untreated, ovariectomized females (Figure 6A), an increase comparable with that observed in ovary-intact females (Figure 3A). In this experiment, circulating PRL was increased 283-fold by E2 treatment (Figure 6B). Although the relative induction of circulating PRL by E2 was greater in ovariectomized animals compared with ovary-intact animals, this resulted entirely from the lower basal PRL levels in the untreated, ovariectomized animals compared with that observed in untreated, ovary-intact animals.

The amount of E2 in trunk blood sera from untreated, ovariectomized ACI rats was <50 pg/ml. The mean level of E2 in trunk blood sera from treated, ovariectomized animals averaged 207 pg/ml (Figure 7).

Discussion

We have demonstrated that E2, administered from s.c. Silastic tubing implants, rapidly induces development of mammary carcinomas in ovary-intact ACI rats. To our knowledge, this is the first demonstration that this naturally occurring estrogen induces development of mammary cancers in this rat strain. Estrogens other than E2 have previously been demonstrated to promote development of mammary cancers in ACI rats. However, the form of estrogen and the manner in which it is administered significantly impact both tumor incidence and latency. For example, in their initial study Dunning *et al.* (8) demonstrated that DES administered from s.c. pellets of compressed crystalline hormone failed to promote mammary cancer development in female ACI rats, whereas this synthetic estrogen administered from s.c. pellets of cholesterol/DES (75:25) promoted development of mammary cancers in 75% of treated animals. In a number of subsequent studies in which DES was used to induce mammary cancers in female ACI rats, the incidence of mammary cancers within the DES-treated populations ranged from 34 to 94% and the median latency to appearance of first tumor ranged from 158 to 425 days (8,10–13,15–18,26). Holtzman *et al.* (17,21) demonstrated the ability of the semi-synthetic estrogen 17α-ethinylestradiol to induce development of mammary cancers in the female ACI rat. In those studies, the latency to appearance of first mammary cancer was similar to that observed in the present study and cancers developed in ~85% of the treated populations (17,21). Estrone has also been demonstrated to induce, in a dose-dependent manner, mammary cancers in female ACI rats (27). However, the incidence of mammary cancers in estrone-treated ACI females ranged from 9 to 18% and the median latency ranged from 218 to 347 days, depending upon the dose of hormone administered (27). Together, these studies strongly suggest that it is the estrogenic activity of these synthetic and naturally occurring compounds, not their chemical properties *per se*, that is responsible for development of mammary cancers. Moreover, the data from the present study indicate that administration of E2 from s.c. Silastic implants is the most effective mode of treatment identified to date for inducing development of mammary cancers in the ACI rat strain.

Also presented herein are data indicating that the ovary modulates susceptibility to estrogen-induced mammary cancers in the female ACI rat. Although the primary purpose of the experiment in which ovariectomized females were treated with E2 for 20 weeks was not to assess the role of the ovary in the etiology of estrogen-induced mammary cancers, it is clear that the ovary-intact females were much more susceptible to the mammary cancer inducing effects of administered E2 than were the ovariectomized females; palpable tumors were observed in ~50% of the ovary-intact animals following 20 weeks E2 treatment, whereas no palpable tumors were observed in ovariectomized females treated with E2 for 20 weeks. At this time we cannot state whether ovariectomy inhibits or simply delays development of mammary cancers in E2-treated ACI rats. In the studies cited above in which mammary cancers

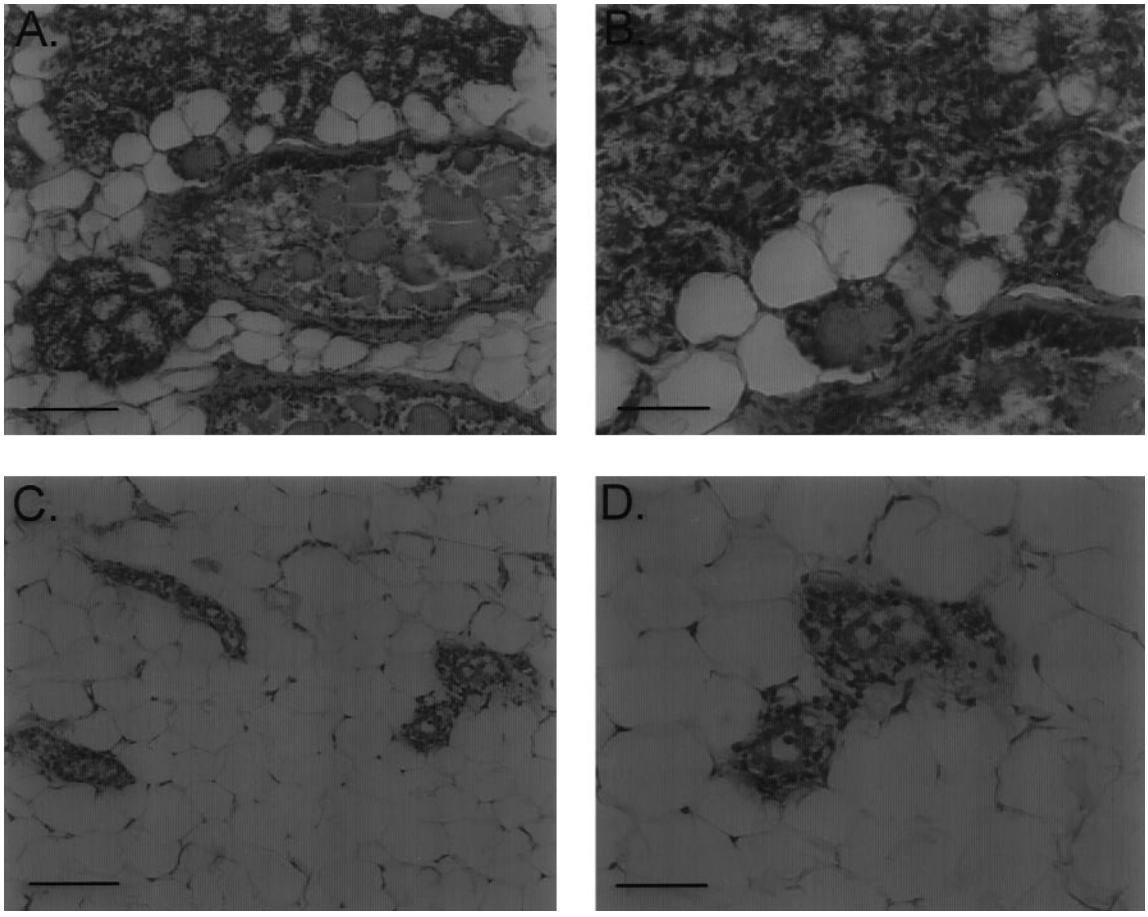

Fig. 5. Histological appearance of mammary tissues from ovariectomized female ACI rats. Treatment of ovariectomized ACI rats with E2 was initiated when the animals were 45 days of age. Control animals received empty Silastic implants. Palpable mammary tumors were not detected in this experiment. Randomly selected regions of grossly normal mammary tissues were processed for histological examination as described in Materials and methods. Representative hyperplastic and normal tissues are illustrated. (**A** and **B**) Grossly normal, but histologically hyperplastic, mammary tissue from a 26.5-week-old, ovariectomized female ACI rat treated with E2 for 20 weeks. (**C** and **D**) Mammary tissue from a 26.5-week-old, ovariectomized female ACI rat not treated with E2. Note the marked atrophy of lobuloalveolar structures. (A) and (C), line indicates 10 μm; (B) and (D), line indicates 5 μm.

were induced in female ACI rats with DES, hormone treatment was initiated in animals whose ages ranged from 50 days to 6 months and age had no apparent effect on tumor incidence or latency. Therefore, we do not believe that the 16–18 day age difference at initiation of E2 treatment, 61–63 days for the ovary-intact females and 45 days for the ovariectomized females, was responsible for the markedly differing results in these two experiments. Ovariectomy essentially abolishes the ability of chemical and physical carcinogens, such as DMBA, NMU and ionizing radiation, to induce development of mammary cancers in the rat. However, treatment with exogenous estrogens essentially reverses the inhibitory effect of ovariectomy (28,29). We interpret these disparate observations to indicate that mammary cancers resulting in ACI rats from treatment with estrogens arise through a fundamentally different mechanism than do the mammary cancers that develop in rats treated with chemical and physical carcinogens.

The differing susceptibilities of ovary-intact and ovariectomized female ACI rats to E2-induced mammary cancers imply that an ovarian factor in addition to E2 is required for rapid development of mammary cancers in the E2-treated female ACI rat. One ovarian factor that may act in this regard is progesterone. Segaloff (12) demonstrated that s.c. implantation of progesterone pellets inhibits mammary cancer development in ovary-intact female ACI rats treated with DES. Both incidence and tumor burden were low in that study, however, making interpretation difficult; five of 14 DES-treated animals developed a total of eight palpable cancers, whereas one of 13 animals treated with DES and progesterone developed two palpable cancers. When administered to Sprague–Dawley rats following treatment with DMBA, progesterone enhanced mammary tumor development (30,31). It is interesting to note that cell proliferation in the breast tissues of human females is greatest during the luteal phase of the menstrual cycle, when the levels of circulating progesterone are maximal (32,33). Therefore, the role of progesterone in estrogen-mediated mammary carcinogenesis in the female ACI rat warrants further investigation.

It has long and often been hypothesized that the large amount of PRL produced by estrogen-induced pituitary tumors plays a direct role in estrogen-mediated mammary carcinogenesis (14,16,34). Data presented herein indicate that susceptibility to mammary cancer development in response to E2 treatment was substantially diminished as a result of ovariectomy, whereas the ability of administered E2 to induce pituitary tumor development and ensuing hyperprolactinemia was similar in ovary-intact and ovariectomized female ACI rats. These data strongly suggest that estrogen-induced hyperprolactinemia is not the sole factor leading to development of mammary cancers in the E2-treated ACI rat. Moreover, many

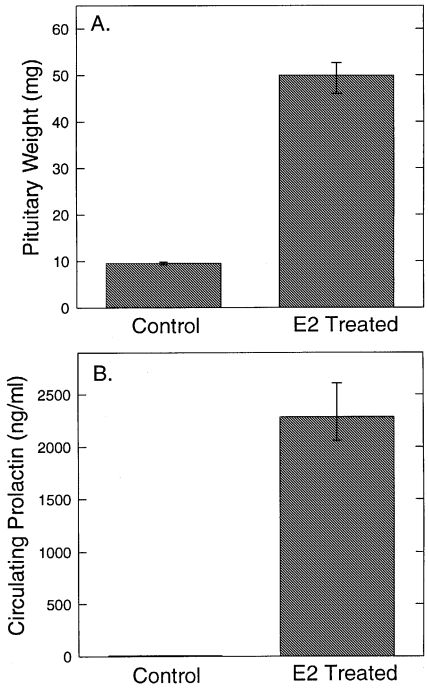

Fig. 6. 17β-Estradiol induces pituitary tumors and hyperprolactinemia in ovariectomized female ACI rats. Animals were treated as described in Figure 5 and Materials and methods. Each data bar represents the mean ± SEM (n = 11 for both E2-treated and untreated). (**A**) Upon sacrifice, the anterior pituitary glands were removed and weighed. (**B**) Trunk blood sera were collected and assayed for PRL by RIA.

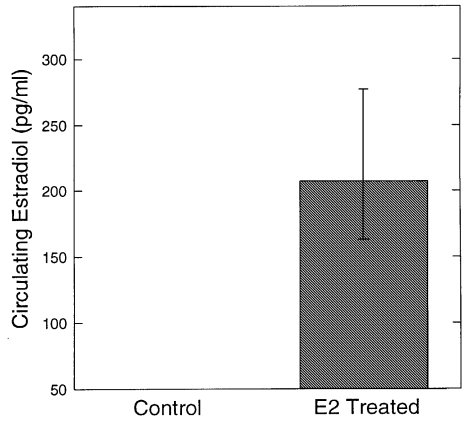

Fig. 7. Level of 17β-estradiol in trunk blood sera from ovariectomized female ACI rats. Trunk blood sera from animals described in Figures 5 and 6 were assayed for E2 by RIA. E2 levels ⩽50 pg/ml could not be accurately measured. Each data bar represents the mean ± SEM.

rat strains develop PRL-producing pituitary tumors when treated with estrogens, yet none of these strains are as highly susceptible to estrogen-induced mammary cancers as the ACI strain.

Although ACI rats rapidly develop mammary cancers when treated with estrogens, they do not appear highly susceptible to other types of cancers. Interestingly, ACI rats are also relatively resistant to spontaneous (8,14–16,20,21), chemically induced (22,23) and radiation-induced (13,16) mammary cancers. We interpret these data to indicate that the gene or genes that contribute to development of estrogen-induced mammary cancers in the ACI rat differ from those genes that contribute to development of spontaneous, carcinogen-induced and radiation-induced mammary cancers. Because of the clear and important role of estrogens in the etiology of human breast cancer, it appears worthy to identify the gene or genes conferring susceptibility to estrogen-induced mammary cancers and to elucidate the mechanisms through which these genes and estrogens act in concert to promote development of mammary cancers.

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