Inhibitory Effects of Estrogen and Castration on the Early Stage of Pancreatic Carcinogenesis in Fischer Rats Treated with Azaserine

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ABSTRACT

Effects of sex steroids on pancreatic carcinogenesis during the early stage were studied in azaserine-treated rats of both sexes. Fischer rats were given weekly i.p. injections of azaserine (30 mg/kg) [CAS:115-02; diazooacetate serine(ester)] at 2 weeks of age and were divided into six groups. Castration, ovariectomy, and s.c. implantations of either a 0.3-mg or a 1.0-mg 17β-estradiol (CAS:50-28-2; estradiol) pellet were performed at 7 weeks of age. The groups were as follows: group 1, intact male; group 2, castrated; group 3, castrated plus 0.3 mg estradiol; group 4, castrated plus 1.0 mg estradiol; group 5, ovariectomized; and group 6, intact female. Rats were killed 4 months after the last injection of azaserine. Azaserine treatment induced atypical acinar cell foci and nodules (AACN) in both sexes. The acidophilic AACN are considered preneoplastic lesions. An apparent sex difference was observed: the number of acidophilic AACN was greater in male rats than in female rats. Castration caused a significant decrease in both the serum testosterone levels and the number of acidophilic AACN, which were comparable to those in ovariectomized female rats. Furthermore, when estradiol treatment was administered to the castrated male rats, a linear decrease in the number of acidophilic AACN and an elevation in the serum estradiol levels were observed and were dose dependent. There were also positive relationships between estradiol treatments and the mean pituitary and pancreas weights.

These results showed that estradiol treatment and the drop in testosterone levels caused by castration were highly effective in inhibiting the development and growth of preneoplastic lesions of the pancreas of the rats treated with azaserine. This estradiol effect was dose dependent. The present study, therefore, provides evidence that estrogen may act as an inhibitor and androgen as a promoter in the early stage of pancreatic carcinogenesis in rats.

INTRODUCTION

Epidemiological studies have shown that the age-adjusted incidence of pancreatic cancer has increased in the United States and several other countries since 1930 and is higher among men than among women (1-3). In rat models for pancreatic carcinogenesis, a sex difference in the incidences of chemically induced acinar cell tumors and preneoplastic lesions has also been reported, and castration caused a lower yield of these pancreatic lesions in male rats (4-6). Furthermore, the growth of a transplantable acinar cell carcinoma originally induced in a Lewis rat by azaserine was more rapid in syngeneic male recipients than in females (7). These studies suggest that pancreatic carcinogenesis may be modified by sex steroids, especially by a male sex hormone. On the other hand, it is obscure whether this sex difference is due solely to a promotive effect of androgen or is due in part to an inhibitory effect of estrogen. In a previous study, the growth of azaserine-induced preneoplastic lesions was decreased by estrogen treatment, but the estrogen dose was far above the physiological level, and the specificity of the effect was questioned (6). The present study was undertaken to clarify this point.

Pancreatic carcinogens are believed to initiate a sequence of focal proliferative cellular changes that progress into grossly visible tumor formation (8, 9). In our laboratory, this sequence has been well characterized in the azaserine-induced rat model (7, 10). AACN3 include both microscopic foci (small lesions) and nodules (large lesions). Foci are detectable 1 to 2 months after azaserine treatment and are considered to be preneoplastic lesions. The AACN were, therefore, evaluated as an indicator for pancreatic carcinogenesis during the early stage in this short term experiment. The quantitative sterological method, as applied to the study of foci in the liver (11, 12) and the pancreas (5, 6, 10), was used for analysis of AACN.

In addition to the measurement of 17β-estradiol (estradiol) and testosterone levels in sera, pituitary glands were evaluated as a biological marker of estradiol action, since estrogens are a potent stimulator of prolactin secretion and cause an enlargement of the pituitary gland and pituitary tumors (13-16). Also, mammary gland growth was evaluated as an indicator for endocrinological status in variously conditioned rats (17).

MATERIALS AND METHODS

Animals. Male and female Fischer (F344) rats were obtained from Charles River Breeding Laboratory, Inc. (Wilmington, MA). Suckling pups were housed in a temperature (21 ± 1°C)- and light (12-h light and 12-h dark cycle)-controlled room with dams. They were fed ad libitum a purified control diet (AIN-76A; Teklad, Madison, WI) without the antioxidant ethosyquin (17) and were given deionized water. At 28 days of age, pups were weaned and randomly divided into six groups, as follows: group 1, intact male; group 2, castrated; group 3, castrated plus 0.3 mg estradiol; group 4, castrated plus 1.0 mg estradiol; group 5, ovariectomized; and group 6, intact female.

Carcinogen, Estrogen, and Surgical Treatments. At 2 and 3 weeks of age, pups received weekly i.p. injections of azaserine (30 mg/kg body weight) [CAS:115-02-6; diazooacetate serine (ester); Calbiochem-Behring, La Jolla, CA] dissolved in 0.9% NaCl solution (3 mg/ml). Castration, ovariectomy, or sham operation were performed at 7 weeks of age under Somonopentyl anesthesia (sodium pentobarbital; The Butler Co., Columbus, OH). Estrogen pellets (designed for 60-day timed release), containing either 0.3 or 1.0 mg estradiol (CAS:50-28-2), and control (placebo) pellets were purchased from Innovative Research of America, (Toledo, OH). Pellets were implanted s.c. on the back of each rat at the time of castration and were replaced every 4 weeks throughout the experiment to maintain continuous estradiol levels. Rats were weighed weekly for the first 2 months and every other week thereafter.

Histological Study. Four months after the last injection of azaserine, all rats were killed. The pancreas, pituitary, and mammary glands, epididymis, uterus, testis, and ovary were removed, weighed, and fixed in Bouin’s solution. Each entire pancreas was divided into two portions, head and tail, by a transection near the superior mesenteric artery and was spread to the largest area in two dimensions before fixation. Paraffin sections were routinely stained with hematoxylin and eosin and examined histologically. Both sections were scanned, and the tail section from each rat was used for quantitative histological analysis. Azaserine-induced AACN were identified, counted, sized, and classified as one of two types, acidophilic or basophilic, according to the criteria

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The abbreviation used are: AACN, atypical acinar cell foci and nodules; Ab W, absolute weight(s); Re W, relative weight(s).

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of Roebuck et al. (10). From the data on AACN observed in focal transection, the number/cm² and diameter (μm) of AACN and volume percent of AACN in pancreas tissue were calculated by using quantitative stereological methods (10, 12).

Measurement of Serum Estradiol and Testosterone Levels. Blood was collected from the axillary veins of all rats at autopsy, under light ether anesthesia. Serum was immediately frozen and stored at −20°C until used for steroid determinations by radioimmunoassay technique, using materials in kit form (Diagnostic Product Co., Los Angeles, CA). Lower limits of sensitivity to estradiol and testosterone were 8 pg/ml and 11 ng/dl, respectively. Each antiserum is highly specific and has a low cross-reactivity to other naturally occurring steroids. The interassay precision of the estradiol and testosterone assays given as coefficient of variation are as follows: estradiol in a control sample, with a mean of 155 pg/ml and 93 separate determinations, was 8.9% and testosterone in a control sample similarly assayed separately 34 times with a mean of 504 ng/dl, gave a coefficient of variation of 9.1%.

Statistical Evaluation. The mean number/cm², diameter, and volume percent of AACN; the mean weights of body, pancreas, and pituitary gland; and the mean steroid levels in the serum were evaluated by analysis of variance tests with multiple comparisons [Scheffe's test (Ref. 18)]. For estradiol and testosterone, values were transformed to the natural log scale to achieve homogeneous variance across experimental groups. The mean epididymis and uterus weights were compared by Student's t test.

RESULTS

Number and Size of AACN. The number and size of acido-philic or basophilic AACN are summarized according to groups in Tables 1 and 2 and Fig. 1. Azaserine treatment induced multiple acido-philic AACN in the pancreas of male and female rats. The mean number of acido-philic AACN/cm² and volume percent of acido-philic AACN in the pancreatic tissue were significantly greater in male rats than in female rats (groups 1 and 6; P < 0.05, P < 0.05, respectively). There was an obvious sex difference in the number of acido-philic AACN. Castration resulted in a marked decrease in both the mean number/cm² (P < 0.05) and volume percent of acido-philic AACN (P < 0.05) in male rats (group 2). The reduced number in castrated male rats (group 2) was comparable to those in ovariec-tomized female rats (group 5). In comparison between female groups 5 and 6, the mean number/cm² and volume percent of acido-philic AACN were increased by ovariec-tomy, but these increases were not statistically significant. The number of acido-philic AACN was affected by the castration, while the effect of ovariec-tomy was not so significant.

Meanwhile, in castrated male groups 2, 3, and 4, effects of estradiol treatment were observed. When either 0.3 or 1.0 mg estradiol was administered, both the mean number/cm² (P = 0.015) and volume percent (P < 0.001) of acido-philic AACN were linearly reduced in castrated male rats. A linear decrease in the mean diameter was also observed but was not statistically significant (P = 0.137). Even 0.3-mg estradiol treatment caused a sharp reduction in the number, to the level in intact female rats (group 6). Estradiol treatment, therefore, dramatically affected the development and growth of acido-philic AACN.

Basophilic AACN. Basophilic AACN developed in all groups treated with azaserine and were mostly small in number and size (Table 2). In contrast to acido-philic AACN, there were no significant differences in the incidence or volume percent between intact male and female rats or hormonally treated rats.

Serum Estradiol and Testosterone Levels. Effects of gonadectomy and estradiol treatment on serum estradiol and testoster-one levels were studied. As shown in Table 3, serum estradiol and testosterone levels in all azaserine-treated rats at autopsy were measured by radioimmunoassay. Estradiol levels in intact female rats (group 6) were higher than those in ovariec-tomized female rats (group 5; P < 0.05). Similarly, testosterone levels in intact male rats (group 1) were between 189 and 375 ng/dl, whereas castration reduced these levels below 11 ng/dl, i.e., to undetectable levels (group 2; P < 0.05). A dose-related elevation of serum estradiol levels was observed in castrated male rats when 0.3 or 1.0 mg estradiol treatment was administered (groups 3 and 4; P < 0.001). Estradiol treatment and ovariec-tomy affected the increase and decrease in circulating estradiol levels, respectively, and castration caused reduction in levels of testosterone.

Body, Pancreas, and Pituitary Weights. The body weight and the Ab W or Re W (mg/100 g body weight) of the pancreas and pituitary gland in all groups are shown in Fig. 2. The mean body weight was significantly decreased in castrated male rats and increased in ovariec-tomized female rats (P < 0.05, P < 0.05) when compared with those in the corresponding control groups. A significant increase in Ab W of pancreas by castration (P < 0.05) and a slight increase by ovariec-tomy were observed. There were no differences in Re W of pancreas. Also, gonadectomy diminished the weights of the epididymis and the uterus from 59.5 ± 3.9 mg to 33.0 ± 1.9 mg (P < 0.001; Student's t test) and from 262 ± 24 mg to 107 ± 23 mg (P < 0.001; Student's t test) in Ab W, respectively.

Among castrated male groups 2, 3, and 4, positive relationships between estradiol treatment and weights of pituitary gland and pancreas were clearly observed and were dose dependent. The pituitary gland in rats given 0.3 or 1.0 mg estradiol were linearly increased in both Re W (P < 0.001) and Ab W (P < 0.001), as compared with those in control rats. A linear increase in Re W of the pancreas (P < 0.001) was also observed in

### Table 1. Number and size of acido-philic AACN in azaserine-treated Fischer rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>Tissue area (mm²)</th>
<th>Diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Observed</td>
<td>Calculated</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Intact male</td>
<td>10</td>
<td>29.0 ± 2.7</td>
<td>524 ± 70</td>
</tr>
<tr>
<td>2</td>
<td>Castrated</td>
<td>10</td>
<td>13.2 ± 2.3</td>
<td>277 ± 75</td>
</tr>
<tr>
<td>3</td>
<td>Castrated + 0.3 mg estradiol</td>
<td>10</td>
<td>13.2 ± 2.3</td>
<td>134 ± 32</td>
</tr>
<tr>
<td>4</td>
<td>Castrated + 1.0 mg estradiol</td>
<td>10</td>
<td>4.0 ± 0.7</td>
<td>102 ± 18</td>
</tr>
<tr>
<td>5</td>
<td>Ovariectomized</td>
<td>11</td>
<td>12.0 ± 2.6</td>
<td>286 ± 55</td>
</tr>
<tr>
<td>6</td>
<td>Intact female</td>
<td>11</td>
<td>8.1 ± 1.6</td>
<td>184 ± 40</td>
</tr>
</tbody>
</table>

* Determined by quantitative stereological methods.
* a ± SE.
* Rats were castrated or ovariec-tomized at 7 weeks of age.
* Different from group 1; P < 0.05 (Scheffe's test).
* Linear regression among groups 2, 3, and 4; P = 0.015 (Scheffe's test).
* Either 0.3- or 1.0-mg estradiol pellets were implanted s.c. on the back and were replaced every 4 weeks.
estradiol-treated groups. Estradiol treatment, therefore, caused the increase in both the pituitary and pancreatic weights, as well as in the serum estradiol levels.

**Histological Observation of Mammary Glands.** The mammary glands of male rats showed a rudimentary structure. In castrated male rats, the mammary gland consisted of poorly developed alveoli in fat pads. In contrast, the mammary glands in castrated male rats receiving estradiol treatment showed active proliferation of alveoli with secretory activity. The retention of secreted substances in distended alveolar lumens was variable in amount. Mammary gland stimulation in castrated male rats receiving estradiol was dose responsive.

**DISCUSSION**

We have shown in this study that estradiol treatment and castration were able to inhibit an early stage of azaserine-induced pancreatic carcinogenesis of rats. In castrated male rats treated with azaserine, both the mean number/cm² and volume percent of acidophilic AACN were reduced to approximately 50% of those in intact male controls. These reduced values were comparable to those in ovariectomized female rats. This finding confirmed recent studies showing that castration following 4-hydroxyaminoquinoline 1-oxide (4) or azaserine (5) administration decreased the incidences of pancreatic tumors and preneoplastic lesions, respectively. Castration caused a fall in testosterone to undetectable levels and a decrease in the body weight and Ab W of pancreas and inhibited the development and growth of microscopically detectable azaserine-induced preneoplastic lesions. The lower number of foci detected in castrated males (group 2) may reflect either the failure of initiated cells to grow to foci of detectable size or an alteration of focal phenotype that precludes detection in hematoxylin- and eosin-stained sections (19).

A dose-dependent inhibitory effect of estradiol treatment on the development of pancreas lesions was also clearly observed in castrated male groups given 0.3 or 1.0 mg estradiol. The lower dose yielded serum estradiol levels in the physiological range. Even this low estradiol dose (group 3), under the conditions of low testosterone levels, reduced these parameters to less than 50% of those in the castrated male controls (group 2). In general, chronic treatment with large doses of estrogens induces reduction in body weight and food consumption, declining tumor development, uterus infection, and higher mortality. Estradiol-treated castrated male rats were smaller than castrated controls but similar to intact female rats. Animals chronically exposed to the continuous stimulus of estrogens, even at low doses, are smaller and their body weight gain is inhibited (15–17, 20). The estrogen doses used, thus, yielded an expected inhibition of growth in our experiment.

Implantation of estrogen pellets induces the development of mammary and pituitary tumors or an enlargement of pituitary glands, consisting of hemorrhagic adenomas or proliferative acidophilic cells in the anterior pituitary gland (13–17). These changes have been extensively suppressed by a potent prolactin suppressor or an antiestrogen (15, 21). We, therefore, estimated the pituitary gland weight as a biological marker of estradiol action in the present study, and a dose-responsive effect of estradiol on the pituitary gland was observed. Furthermore, hormonal status in the estradiol-treated rats was reflected in the mammary gland growth. Functional mammary gland growth was considered to result from an interaction of estrogen and prolactin (15, 17). These biological, endocrinological, and morphological findings indicated that estradiol had acted appropriately at each dose as an estrogenic agent in responsive tissues. Therefore, the effects demonstrated in the pancreas have been considered to be related to an estradiol effect that might be either direct or indirect.

While azaserine is a directly acting mutagen in the *Salmonella* test system, its activity seems dependent on pyridoxal-dependent enzymes, but not cytochrome P-450 (7, 22, 23). Estradiol treatment and castration were administered 5 weeks after the last injection of azaserine. Therefore, initiation events were completed before manipulation of endogenous or exogenous sex hormones. Consequently, modulation by sex steroids in our experiment was apparently due to an effect during the promotion phase of carcinogenesis.
ESTROGEN AND PANCREATIC CARCINOGENESIS

Interestingly, there was a dose-related increase in Re W of the pancreas in estradiol-treated groups, which is in contrast to the fact that the relative weights in male and female rats were not affected by gonadectomy. Mori et al. (24) have shown an increase in the pancreas weights of mice with hyperprolactinemia, indicating the possible participation of prolactin in pancreatic tumorigenesis in mice by stimulating acinar cells. Prolactin stimulates insulin secretion (25). An anatomical interrelation in blood supply pattern and capillary anastomoses and an intimate relationship in simultaneous proliferation between endocrine and exocrine pancreas under special conditions have been reported (26, 27). Estradiol treatment, therefore, might have affected normal pancreas acinar cell growth indirectly via prolactin.

Although the mechanism of estradiol action in our experiment is uncertain, a dual role of estradiol is suggested. Estrogen acts directly on its target tissues such as the uterus, mammary gland, and pituitary gland through estrogen receptors (28–30). Although the pancreas has not been widely recognized as a steroid target tissue, a steroid-binding protein has been shown to exist in normal pancreas of several species, human fetal pancreas, and human pancreatic carcinoma (31–35). These indicate the presence of a steroid receptor, although its function is unknown. Therefore, estradiol may exert its inhibitory effect on the growth of AACN by acting directly in the acinar cells. On the other hand, we cannot exclude the possibility of an involvement of prolactin in the action of estrogen under special conditions, which may lead initiated acinar cells into a more differentiated or dormant status. Prolactin is the principal hormone in the promotion step of rat mammary tumorigenesis (15, 21, 36–39). Nevertheless, lactation suppresses this process, in spite of higher prolactin levels (40–42). In addition, castration might have contributed by causing changes in the overall hormonal balance of host rats, such as an abnormal production of gonadotropin in castrated males. Finally, a role for a non-specific mechanism such as reduced caloric intake cannot be excluded.

The present study provides evidence that azaserine-induced rat pancreatic carcinogenesis during the early stage can be modulated by sex hormones. While an apparent sex difference in the preneoplastic lesions of the pancreas was detected, there was no significant difference between ovariectomized and intact female rats. This finding may be partly explained by the loss of endogenous testosterone in ovariectomized females. The sex difference in pancreatic carcinogenesis may be due to both a promotive effect of androgen and an inhibitory effect of estrogen.

Furthermore, estradiol treatment recently has been observed to inhibit the growth of a transplantable acinar cell carcinoma of the pancreas, originally induced in a rat by azaserine (43). The azaserine-induced rat model system, thus, seems to be a useful tool for studies of the mechanism of pancreas tumor response to sex hormones. Further study is required to determine the mechanisms of sex steroid action in the pancreas and, perhaps, to establish a rationale for new therapeutic approaches, as suggested by Greenway (44).

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