Mechanism of Estrogen Enhancement in the Growth of Androgen-dependent Shionogi Carcinoma 115

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ABSTRACT

The mechanism of estrogen enhancement in the growth of androgen-dependent Shionogi carcinoma 115 (SC115) maintained in castrated DS mice by low doses of androgen (10 µg of testosterone propionate or 4 µg of 5α-dihydrotestosterone/mouse/day) is reported. Although the low androgen treatment slightly but significantly (P < 0.05) stimulated tumor growth, concomitant estrogen (4 µg of 17β-estradiol/mouse/day) significantly (P < 0.01) enhanced the tumor growth. The high growth rate, histological type (medullary carcinoma), androgen dependency, and high androgen receptor content of the tumor grown during estrogen plus low androgen treatment did not differ significantly from those of the original SC115 tumor grown in normal males or in castrated mice treated with high doses of androgen. On the other hand, the treatment with low doses of androgen alone induced the development of slowly growing spindle-shaped cells from the medullary SC115 cells. The spindle-shaped cells containing low levels of androgen receptor were shown to be androgen independent and were also induced from the SC115 cells in nontreated castrated mice. These findings demonstrated that low doses of androgen and estrogen synergize to maintain and increase the growth of SC115 cells, whereas low doses of androgen alone fail to maintain the SC115 cells.

INTRODUCTION

Androgen-dependent mouse mammary carcinoma SC115 was established in 1964 by Minesita and Yamaguchi (1, 2). The original tumor arose spontaneously as an androgen-independent adenocarcinoma of mammary origin in a female DS mouse, and it grew equally well when transplanted to male and female mice. After passage in male DS mice for 19 generations, the tumor was found to be androgen dependent, defined by its failure to grow in either female or castrated male mice and by its ability to grow in female or castrated male mice given androgens. Cells derived from this tumor retain their androgen responsiveness in vivo (3–5) and in cell culture (6–9). We (4) and other investigators (3, 5–9) have reported that androgen activity on the stimulation of SC115 tumor growth is mediated by a specific AR system in SC115 cells.

We demonstrated in 1970 (10) that grafted SC115 tumor did not incorporate a significant amount of radioactivity following a s.c. injection of [3H]17β-estradiol into the host animals. On the other hand, Jung-Testas et al. (7) and King et al. (8) in 1976 demonstrated ER in SC115 cells in culture. We (11) in 1978 and other investigators (12) in 1982 also found ER in cytosols obtained from S.C. grafted SC115 tumors. Although other investigators (7, 8, 12) found ER in SC115 cells, they supported our original findings (2) that estrogen was unable to stimulate the proliferation of SC115 cells. In 1984, however, we (13) demonstrated for the first time the stimulative effect of estrogen on the growth of androgen-dependent SC115 tumor in vivo. These findings suggest that the response to 17β-estradiol and the ER content in the SC115 tumors have increased over the past 20 years.

In our recent studies (13), addition of 4 µg (per mouse per day) of 17β-estradiol to 10 µg of TP significantly enhanced the growth of SC115 tumor in castrated mice. The addition of lower or physiological doses of estrogen (1 µg of 17β-estradiol/mouse/day or estrogens secreted from the ovaries of normal adult female mouse) to 10 µg of TP was ineffective. Since the minimum effective dose of 17β-estradiol (4 µg/mouse/day) was less than that of TP (10 µg/mouse/day) and since the prolactin-mediated estrogen activity could be ruled out (administration of bromocriptine with estrogen had no effect on the estrogen-enhanced tumor growth), the estradiol enhancement of the growth of SC115 tumor seems to be mediated by typical ER demonstrated in the tumor (13). In the present paper, we present the mechanisms of estrogen enhancement in the growth of androgen-dependent SC115 tumor maintained in castrated mice by low doses of androgen.

MATERIALS AND METHODS

Animals and Tumors. Two- to 3-month-old male DS mice raised in our laboratory were used. When castrated mice were used, the mice were castrated 1 week before the implantation of seed tumors or the experiment, unless specified otherwise. A fragment of tumor (about 1 mm) from normal males or in castrated mice treated with high doses of androgen. On the other hand, the treatment with low doses of androgen alone induced the development of slowly growing spindle-shaped cells from the medullary SC115 cells. The spindle-shaped cells containing low levels of androgen receptor were shown to be androgen independent and were also induced from the SC115 cells in nontreated castrated mice. These findings demonstrated that low doses of androgen and estrogen synergize to maintain and increase the growth of SC115 cells, whereas low doses of androgen alone fail to maintain the SC115 cells.

2 To whom requests for reprints should be addressed.
3 The abbreviations used are: SC115, Shionogi carcinoma 115; AR, androgen receptor; ER, estrogen receptor; DHT, 5α-dihydrotestosterone; TP, testosterone propionate; TEDM, 10 mM Tris, 1.5 mM EDTA, 0.5 mM dithiothreitol, and 10 mM sodium molybdate, pH 7.4 at 20°C.
4 Received 12/3/84; revised 7/1/85; accepted 7/3/85.
mice were given injections of 0.05 ml of vehicle.

**Determination of Tumor Growth.** Length and width of each tumor was measured once/week, and the mean of the length and width was used as an index of tumor size.

ER and AR in Tumor Cytosol. Methods for ER and AR assays were similar to those used in our previous study (13). For the examination of ER and AR, tumors (1-2 cm in diameter) induced from seed SC115 tumors in castrated mice treated with or without 10 or 100 nM of TP and/or 4 mg of 17β-estradiol and in normal male mice were used. Mice were sacrificed 24 h after the last injection of TP and/or 17β-estradiol or the castration. All procedures for ER and AR assays were carried out at 0-4°C. After removal of necrotic and connective tissues, the tumors were minced and homogenized in 6 volumes of TEDM buffer using a Polytron PT 10 homogenizer (Brinkmann Instruments, Inc., Westbury, NY) set at 4, with three 10-s runs, allowing 30 s for cooling between each run. The homogenate was centrifuged at 105,000 x g for 60 min, and the supernatant cytosol was obtained without the superficial lipid layer. For ER assay, the cytosol in the TEDM buffer was incubated with 0.1-10 nM of [3H]estradiol in the presence of a 250-fold molar excess of unlabeled DHT for 20 h (total reaction volume, 0.4 ml). The unlabeled DHT was included for the estimation of the 17β-[3H]estradiol binding sites, since 17β-[3H]estradiol bound to AR with relatively low but significant affinity in the tumor cytosol (4, 11). Parallel incubations were carried out with 17β-[3H]estradiol plus DHT in the presence of a 250-fold molar excess of unlabeled diethylstilbestrol to obtain the nonspecific binding values. For AR assay, the cytosol in the TEDM buffer was incubated with 0.1-10 nM of [3H]DHT for 20 h in 0.4 ml. Parallel incubations were carried out with [3H]DHT in the presence of a 250-fold molar excess of DHT. After incubation for ER and AR assays, 0.4 ml of a suspension of 10 mg Tris:1.5 mM EDTA:0.5% Norit A:0.005% Dextran T-70 (pH 8.0) at 20°C was added to each tube, followed by incubation for 30 min with intermittent vortexing. The tubes were centrifuged at 2000 x g for 10 min, and the aliquots (0.2 ml) of the supernatants were counted. The difference between the total and nonspecific binding values was considered the specific binding value. The maximum binding sites for the specific binding value and Kd were estimated by Scatchard plot analyses (14).

**Retention of [3H]Testosterone and [3H]DHT in Cytosol and Purified Nuclei of Tumor following Injection of [3H]Testosterone.** Tumors (1-2 cm in diameter) were obtained 3 and 6 weeks, respectively, after the transplantation of seed SC115 tumors into castrated mice injected daily with 10 nM of TP plus 4 μg of 17β-estradiol or 10 μg of TP. Twenty-four h after the last injection of TP with or without 17β-estradiol, [3H]testosterone (40 μCi; 416 pmoles/mouse) was injected s.c. into the tumor-bearing mice. The mice, 4 in each group, were sacrificed 20 min and 1, 3, and 9 h after the injection of [3H]testosterone, and the tumors were obtained. For the estimation of nonspecific retention value, 100 μg of unlabeled TP was injected s.c. into mice, 4 in each group, at 1 and 2 h before the injection of [3H]testosterone.

For the preparation of cytosol and purified nuclei, our previous method was used (15, 16). In short, tumors were homogenized and separated into crude nuclear and supernatant fractions. The purified nuclei were prepared from the crude nuclear fraction using a discontinuous sucrose density gradient method, as reported previously (15). Recovery for the purification procedure was estimated by DNA determination. The cytosol fraction was obtained from supernatant fraction from crude nuclear separation.

Radioactive metabolites and [3H]testosterone in the cytosol and purified nuclear fractions were extracted and separated into polar metabolites, testosterone, and DHT fractions by paper chromatography, as reported previously (16). Finally, radioactive steroids in the testosterone and DHT fractions were recrystallized with 15 mg of testosterone and DHT, respectively, to constant specific activity, in order to obtain accurately the amounts of both radioactive androgens present in all samples. The amounts of [3H]testosterone and [3H]DHT were corrected for the loss during purification procedures. The difference between the total and nonspecific retention values for testosterone and DHT was considered the specific retention value.

**Metabolism in Vivo of [3H]Testosterone in Tumor.** Male mice bearing SC115 tumors were castrated 3-4 weeks after grafting. The castrated mice, 4 in each group, were given injections daily with 10 μg of TP or 10 μg of TP plus 4 μg of 17β-estradiol for 2 days starting from the day of castration. Twenty-four h after the last injection, [3H]testosterone (40 μCi; 416 pmol/mouse) mixed with 10 μg of testosterone or 10 μg of DHT plus 4 μg of 17β-estradiol was injected s.c. into the mice; the mice were sacrificed 1 h later. About 300-500 mg of tumors were homogenized, and radioactive steroids in the homogenates were extracted. The radioactive steroids in the extracts were separated into polar (origin) fraction and 2 non-polar fractions (testosterone plus 5α-androstaned-3α,17β-diol and the other 4 steroids shown in Table 5) by paper chromatography in a hexane:benzene:1:1 (v/v)-formamide system. Metabolites in the non-polar fractions were separated into each steroid, as described previously (17). Finally, except for polar metabolites, the separated radioactive steroids were recrystallized with 15 mg of nonradioactive standard steroids to constant specific activity in order to accurately estimate their amounts.

**Estimation of Testosterone.** The method for the estimation of serum testosterone by radioimmunoassay has been described in detail (18). The intra- and interassay coefficients of variation in male serum obtained from 10 assays were 8.5 and 10.9%, respectively.

**Miscellaneous Assays.** The protein and DNA concentrations were determined according to the methods of Lowry et al. (19) and Burton (20), respectively.

**Histology.** Tumors were fixed in 10% buffered formalin (pH 7.2), embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Although the original SC115 tumor is an undifferentiated medullary carcinoma showing a compact cell pattern, we have already described the development of androgen-independent spindle-shaped cells from the original SC115 cells after removal of androgen from the host (4, 21). The tumor was designated "medulIary type" when more than 90% of the tumor was composed of undifferentiated medulIary cells, "spindle cell type" when more than 90% of the tumor was composed of spindle-shaped cells, and "mixed type" when 90% or less of the tumor was composed of either medulIary or spindle-shaped cells alone.

**Statistical Methods.** Tumor size, levels of AR and ER, incorporation of [3H]-labeled androgens, and serum testosterone levels were compared by Student's t-test (22).

**RESULTS**

**Stimulative Effects of 17β-Estradiol on Tumor Growth.** Seed SC115 tumors were transplanted into castrated mice injected daily with 10 μg of TP, 4 μg of 17β-estradiol, 10 μg of TP plus 4 μg of 17β-estradiol, or 100 μg of TP per mouse. The seed tumors were also transplanted in normal male mice. In the castrated mice, the tumors grew very slowly when only the vehicle was injected, but 10 μg TP treatment alone or 4 μg 17β-estradiol treatment alone slightly but significantly (P < 0.05) stimulated the tumor growth. Addition of 4 μg of 17β-estradiol to 10 μg of TP significantly (P < 0.01) enhanced the tumor growth (Chart 1, upper panel), and the growth rate approached the levels found in the normal males and in the castrated males treated with 100 μg of TP.

In the castrated mice, daily injections of 4 μg DHT per mouse stimulated the tumor growth slightly, and the growth rate reached the level induced by 10 μg of TP. The addition of daily injections of 4 μg 17β-estradiol per mouse to 4 μg of DHT significantly (P < 0.01) enhanced the tumor growth (Chart 1, lower panel). The findings demonstrate that androgen, either testosterone or DHT,
Treated with a Low Dose of TP or a Low Dose of TP plus 17β-Estradiol. The high growth rate and histological type (medullary carcinoma) of tumors grown in castrated mice treated daily with a low dose of TP (10 μg/mouse/day) plus 17β-estradiol (4 μg/mouse/day) starting from the day of transplantation of seed SC115 tumors (TP and 17β-estradiol tumor) did not differ significantly from those of the original SC115 tumors grown in normal male mice. However, the low growth rate and histological type (appearance of spindle-shaped cells) of tumors grown in castrated mice treated with 10 μg of TP alone (TP tumor) were similar to those of androgen-independent tumors developed from the original SC115 tumors after androgen removal. We already demonstrated the transformation of androgen-independent spindle-shaped cells from the original SC115 cells after removal of androgen from the host, since both types of cells had 6 identical chromosome abnormalities (4, 21). Therefore, we examined the androgen dependency of the TP tumors and the TP and 17β-estradiol tumors grown in castrated mice.

Seed tumors from the TP tumors or the TP and 17β-estradiol tumors were transplanted in male and female DS mice. The TP and 17β-estradiol tumors could rapidly grow only in the males but not in the females, while the growth rate of the TP tumors in both males and females was almost the same and was evidently slower than that of the TP and 17β-estradiol tumors in the male mice (Chart 2).

AR and ER in Tumors in Castrated Mice Treated with or without TP and/or 17β-Estradiol. Tumors grown from seed SC115 tumors in castrated males treated with 10 μg of TP plus 4 μg of 17β-estradiol were found to be androgen-dependent medullary carcinoma similar to the original SC115 tumor, whereas tumors grown in castrated males treated with 10 μg of TP alone were androgen independent and consisted predominantly of spindle-shaped cells (similar to the tumors grown in castrated mice). We reported already that androgen-independent sublines that consisted only of spindle-shaped cells contained no or smaller amounts of AR than did the original SC115 tumor (4, 16, 21).

Tumors for AR and ER assays were obtained 24 h after castration or the last injection of TP and/or 17β-estradiol. AR and ER were found in the cytosols of all the tumors grown in normal males and in treated and nontreated castrated males. As shown in Table 2, concentrations (fmol/mg protein) of AR in the tumor cytosols were inversely related to the relative amounts of androgen synergize to increase growth rate of the SC115 tumor and that DHT is the more effective androgen.

Histology of Tumors in Castrated Mice Treated with or without TP and/or 17β-Estradiol. The morphology of tumors grown from seed SC115 tumors depends on the hormonal environment of the hosts. The tumors were composed of medullary cells when the tumor growth in castrates was stimulated by daily injections of 100 μg of TP, whereas the tumors consisted of spindle cells when the castrates were injected with only vehicle. When seed SC115 tumors grew in castrated mice given injections of 10 μg of TP or 4 μg of 17β-estradiol, about one-half of the tumors were of spindle cell type and the others were of mixed type. In contrast, most of the tumors were medullary type when 4 μg of 17β-estradiol was added to 10 μg of TP (Table 1). The original SC115 tumors grown in normal male mice were composed of medullary cells (Table 1). The findings suggest that in castrated mice, daily injections of 100 μg of TP or 10 μg TP plus 4 μg of 17β-estradiol are able to maintain and stimulate the growth of SC115 cells, whereas daily injections of 10 μg TP alone fail to maintain the SC115 cells and induce the development of androgen-independent spindle-shaped cells.

Androgen Dependency of Tumors Grown in Castrated Mice

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spindle-shaped cells found in the tumors. Therefore, concentrations of AR in the tumors grown by 10 μg of TP plus 4 μg of 17β-estradiol were similar to those in the SC115 tumors grown in normal males or in castrated males treated with 100 μg of TP but were higher than those in the tumors grown by 10 μg of TP alone. When AR were expressed as fmol per mg DNA of the tumors, results were the same (Table 2). There were no significant differences in Kₐ for AR or ER among the tumors grown under various hormonal conditions.

In the next experiment, we examined the specific retention values (difference between total and nonspecific retention values) of testosterone and DHT following injection of [³H]testosterone in cytosols and nuclei of tumors developed in castrated mice treated daily with 10 μg of TP or 10 μg of TP plus 4 μg of 17β-estradiol (Table 3). The specific retention values in the cytosol and nuclei 20 min after injection of [³H]testosterone were again about 3-fold higher (P < 0.01) in the tumors treated with 10 μg of TP plus 4 μg of 17β-estradiol than in the tumors treated with 10 μg of TP alone. Although the specific retention values in the cytosol and nuclei significantly decreased 1 h after injection, there were similar 3-fold differences in the amount of testosterone or DHT between these tumors (data not shown). Testosterone (data not shown).

The results shown in Tables 2 and 3 are valid only if all circulating androgen was eliminated before the experiments were carried out. Since testosterone was administered as the propionate in a vehicle containing carboxymethylcellulose, this may not have been achieved in the 24-h period allowed. However, serum testosterone levels, which were high 20 min after the injection of 10 μg TP, decreased rather rapidly and approached the preinjection level 8 h after injection. Furthermore, no significant effect of 17β-estradiol on serum testosterone levels after the TP injection was demonstrated. These results suggest that almost all circulating testosterone, if not all, was eliminated within 24 h after the injection of 10 μg TP with or without 17β-estradiol (Table 4).

Effect of 17β-Estradiol on in Vivo Metabolism of [³H]Testosterone in SC115 Tumor. Consideration of the data of Chart 1 showing the greater effectiveness of DHT and the known ability of 17β-estradiol to inhibit testosterone metabolism by competition for 17β-ol-dehydrogenase suggest that an effect of estrogen may be to inhibit testosterone metabolism and thereby increase the intracellular concentrations of both testosterone and DHT in SC115 tumors. However, we failed to find such effects of 17β-estradiol on testosterone metabolism in these tumors on the third day of treatment (Table 5). Again, testosterone was the major intracellular androgen in the SC115 tumors regardless of estrogen. No significant increase in 5α-reductase activity was demonstrated in the presence of 17β-estradiol (Table 5).

DISCUSSION

The present findings demonstrate that relatively high doses of estrogen and low doses of androgen synergize to maintain and...
increase the growth of androgen-dependent SC115 cells, whereas low doses of androgen alone fail to maintain the SC115 cells. In the presence of low doses of androgen and 17β-estradiol, tumors grown from seed SC115 tumors showed high growth rate, high AR content, histological type of medullary carcinoma, and androgen dependency, which did not differ significantly from those of the original SC115 tumor grown in normal males. In the presence of low doses of androgen alone, however, slowly growing androgen independent spindle-shaped cells having lower levels of AR were induced from the SC115 cells. It was reported that estrogen enhances androgen-induced prostate growth in castrated dogs to a degree comparable to that in intact males.

It was found that estrogen stimulates the growth of epithelial cells from human metastatic prostate tumor in vitro in the presence of DHT, although DHT alone does not stimulate the growth of these cells (28). The administration of estrogen increases AR content in human prostate cancer (29). In contrast to the important roles of androgens, however, the effects of estrogens on the development and growth of prostatic hypertrophy and cancer in humans are not clear. The SC115 tumor seems to be a good model for elucidating the stimulative effect of estrogens on the growth of androgen-dependent tissues and tumors.

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REFERENCES

ESTROGEN AND ANDROGEN-DEPENDENT TUMOR


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