Jpn. J. Cancer Res. 83, 995–1001, September 1992

Maintenance of Androgen-, Glucocorticoid- or Estrogen-responsive Growth in Shionogi Carcinoma 115 Subline Sustained in Castrated Mice with High Dose of Estrogen for 30 Generations (3 Years)

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Shionogi carcinoma 115 (SC115), an androgen-dependent mouse mammary tumor, rapidly loses its androgen responsiveness after androgen withdrawal. The growth of this tumor can also be stimulated by high doses of estrogen or glucocorticoid. In the present study, the maintenance of hormone-responsive growth of SC115 tumors with a high dose of estrogen was examined in castrated male mice using an SC115 subline obtained by serial transplantations of SC115 tumors in estrogen-treated castrated mice for 3 years (30 generations) (subline E_2). Seed tumors from both SC115 and subline E_2 could rapidly grow in castrated mice given daily injections of testosterone propionate (TP), 17β-estradiol (E_2), or dexamethasone (Dex) (100 μg/mouse/day) but not in those given vehicle alone. Although SC115 and subline-E_2 tumors grown with TP or Dex showed temporary regression after steroid withdrawal, the tumors grown with E_2 did not show such temporary regression. The TP-, E_2-, or Dex-induced growth of subline-E_2 tumors was almost the same as that of the original SC115 tumors. However, responsiveness to androgen, estrogen or glucocorticoid of both tumors disappeared within one passage in steroid-depleted castrated mice. The present findings demonstrate that the loss of responsiveness to androgen as well as to high doses of estrogen or glucocorticoid of SC115 tumors can be prevented in castrated mice not only with androgen but also with high doses of estrogen.

Key words: Shionogi carcinoma 115 — Mouse mammary tumor — Androgen — Estrogen

Shionogi carcinoma 115 (SC 115[7]),[1] an androgen-responsive mouse mammary carcinoma which was established in 1964,[2] had been thought to be stimulated into growth only by androgens *in vivo*[1,3-6] and in cell culture.[7-11] This conclusion is reasonable when the physiological concentrations of hormones are considered, since SC115 tumor does not grow in normal adult female mice. However, recent studies have shown that the growth of SC115 cells is also stimulated by high, but not physiological, doses of glucocorticoids both *in vivo*[12] and in cell culture[12-15] and by high, but not physiological, doses of estorogens only *in vivo*.[16,17] These growth-stimulatory effects of androgens, glucocorticoids and estrogens are mediated through AR, GR and ER, respectively.[1,4-17] Recently Luthy *et al.*[18] found that high doses of E_2 stimulated the growth of a highly androgen-sensitive clone of SC115 cells in cell culture, and they have also shown that E_2 acted through AR.

In the absence of androgen *in vivo*[1,19] and in cell culture,[7,13,20,21] there is a rapid increase in autonomous cancer cells formed from SC115 cells. However, the increase can be markedly delayed by androgen addition. Recently, it was demonstrated that glucocorticoids can also delay the loss of androgen responsiveness of SC115 cells *in vivo*[12] and in cell culture.[22] Darbre and King[23,24] very recently found that androgen protects via AR against loss of glucocorticoid-sensitive growth of SC115 cells in culture and glucocorticoid protects via GR against loss of androgen-sensitive growth, and that loss of the response occurs despite the presence of fully functional receptors in SC115 cells as determined by transfection experiments. Therefore, the post receptor defect appears to be at the level of the glucocorticoid and androgen response element of the responsive genes in SC-115 cells and may involve DNA methylation; the same response element has been shown to mediate induction by

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[7] Abbreviations; SC115, Shionogi carcinoma 115; subline-E_2, SC115 subline obtained by serial transplantations of SC115 tumors in E_2 (high dose, 100 μg/mouse/day)-treated castrated mice for 3 years (30 generations); AR, androgen receptor; ER, estrogen receptor; GR, glucocorticoid receptor; TP, testosterone propionate; E_2, 17β-estradiol; Dex, dexamethasone; MMTV, mouse mammary tumor virus; LTR, long terminal repeat; FGF, fibroblast growth factor.

GR-glucocorticoid as well as AR-androgen complexes.[25] In the present study, the possibility that high doses of estrogen can prevent or delay the loss of androgen responsiveness of SC115 cells was investigated using castrated mice.

MATERIALS AND METHODS

Animals and tumors Male DS mice (2–3 months old), raised in our laboratory, were used. When seed tumors were grafted in castrated mice, the castration was carried out at least one week in advance. A fragment of tumor (1 mm^3) was inserted beneath the dorsal skin, using a specially devised needle.[2] Seed tumors of SC115 were obtained from generations 348 to 375. The SC115 tumors were maintained in adult male DS mice. Seed tumors of subline E_2 were obtained from generations of 29 to 40. The subline-E_2 tumors were produced by serial transplantations of SC115 tumors for 3 years in E_2 (100 μg/day)-injected castrated male mice. These seed tumors were obtained when SC115 and subline-E_2 tumors reached 1.5–3 cm in diameter.

Injections of steroids TP, Dex, or E_2 (100 μg) was suspended in 0.05 ml of vehicle (saline, 0.4% polysorbate 80, 0.5% carboxymethylcellulose, and 0.9% benzyl alcohol) for s.c. injection. Control mice received injections of 0.05 ml of vehicle.

Determination of tumor growth The length and width of each tumor were measured, and the mean of the two values was used as an index of tumor size.

Tumor growth in response to androgen or high dose of estrogen or glucocorticoid *in vivo* To examine tumor growth in response to physiological doses of androgens, seed tumors from SC115 or subline E_2 were grafted in 6 normal males and 6 castrated males, and tumor size was measured once a week for 3 to 10 weeks. When tumor growth in response to androgen or a high dose of estrogen or glucocorticoid was examined, seed tumors from both tumors were grafted in castrated males given vehicle, TP, E_2, or Dex (100 μg/mouse/day). Daily injections of TP, E_2, or Dex were given during the entire experimental period, on days 1–14 and on days 1–3 after grafting, since we had previously found that these TP treatments result in continuous growth, temporary regression and no growth of SC115 tumors, respectively.[1] Tumor size was measured approximately once a week for five weeks.

Assay of AR, ER, and GR in tumor cytosol SC115 and subline-E_2 tumors were removed 24 h after the castration and the last injection of E_2, respectively. For GR assay, adrenalectomy was carried out 4 weeks before killing; the adrenalectomized mice were given saline instead of water. Steroid receptors in tumor cytosols were assayed as described previously.[12, 16] In short, [1,2,6,7-^{3}H]testosterone (102 Ci/mmol), [1,2,6,7-^{3}H]E_2 (93 Ci/mmol), and [6,7-^{3}H]Dex (38 Ci/mmol) obtained from New England Nuclear (Boston, MA) were used for AR, ER, and GR assays, respectively. Bound and free steroids were separated by the hydroxylapatite method. The maximum numbers of binding sites and K_d values were calculated by the procedure of Scatchard.

Statistical methods Values (tumor size) were compared by using Students's t test.[26]

RESULTS

Protective effect of a high dose of E_2 given in castrated mice for one generation on loss of androgen-responsive growth of SC115 tumors In preliminary experiments, we examined whether SC115 tumors grown in castrated mice with a high dose of estrogen for one generation still retained their androgen responsiveness. SC115 tumors were transplanted into castrated mice given TP, a high dose of E_2, or vehicle. As shown in Figs. 1 and 2, seed tumors from castrated mice given TP as well as E_2 could grow rapidly and significantly only in normal males but not in castrated males. The androgen-responsive growth of tumors grown with 100 μg of E_2 for one generation was essentially the same as that of the original SC115 tumors. However, the growth rate of seed tumors from

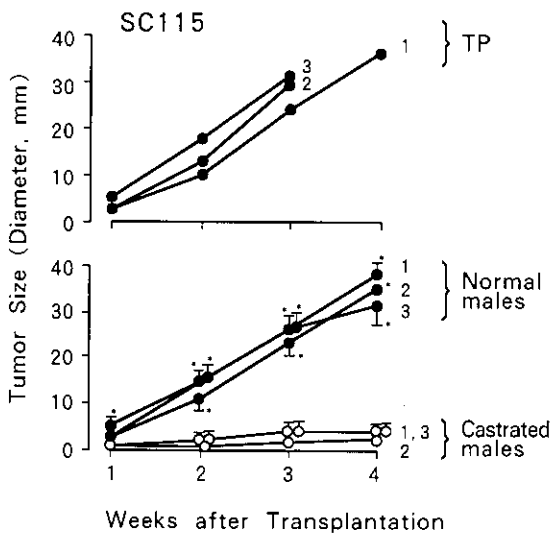

Fig. 1. Androgen responsiveness of SC115 tumors grown in castrated mice with TP for one generation. SC115 tumors were transplanted into 3 castrated male mice given daily injections of 100 μg of TP (1–3, top). Seed tumors from each mouse (1–3, top) were transplanted into a group of 6 normal (●) and a group of 6 castrated (○) male mice (1–3, bottom); values are the means±SE of 6 mice of each group. * $P<0.01$, when compared to castrated males.

castrated males given only vehicle was almost the same in both normal and castrated males, and was evidently slower than that of the original SC115 tumors in normal males (Fig. 3). These results demonstrate that androgen responsiveness of SC115 tumors can be maintained for at least one generation by high doses of estrogens, as is the case with androgens, but the androgen responsiveness disappears within one passage in steroid-depleted mice. Therefore, we next examined whether subline-E_2 tumors sustained in castrated mice with a high dose of E_2 for 3 years still retained androgen responsiveness; maintenance of responsiveness to a high dose of E_2 or Dex in the subline-E_2 tumors was also investigated.

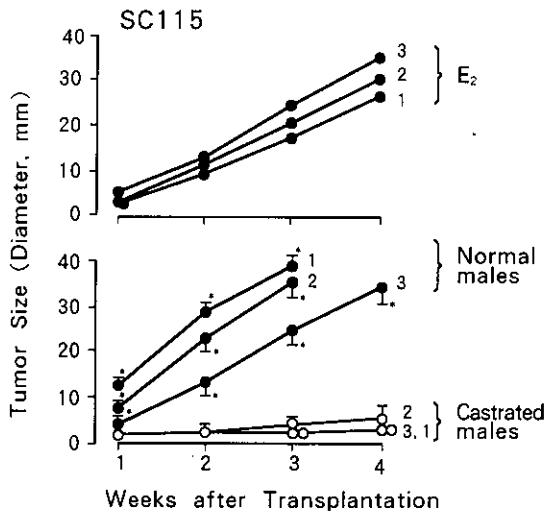

Fig. 2. Androgen responsiveness of SC115 tumors grown in castrated mice with a high dose of E_2 for one generation. Except for the treatment of castrated mice with daily injections of 100 μg of E_2, the treatments were the same as those for Fig. 1. * $P<0.01$, when compared to castrated males.

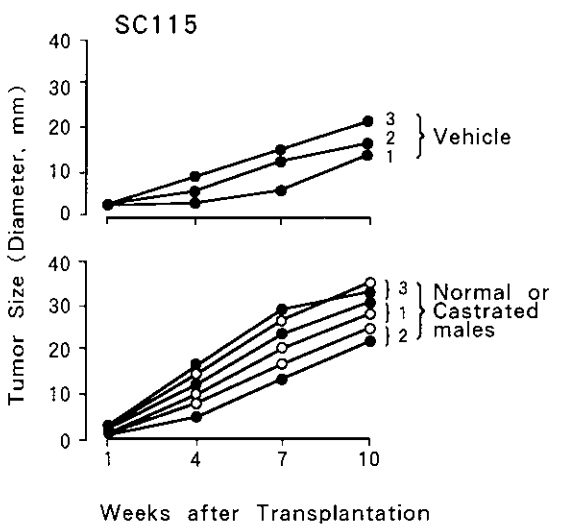

Fig. 3. Loss of androgen responsiveness of SC115 tumors slowly grown in castrated mice for one generation. Except for the treatment of castrated mice with vehicle alone, the treatments were the same as those for Fig. 1.

Maintenance of androgen-, glucocorticoid-, or estrogen-responsive growth in SC115 subline (subline-E_2) sustained in castrated mice with a high dose of E_2 for 3 years (30 generations) Figs. 4–6 show the effects of TP or a high dose of E_2 or Dex on the growth of subline-E_2 and SC115 tumors in castrated mice.

When steroids were given during the entire experimental period, seed tumors from both SC115 and subline E_2 could rapidly grow in castrated mice given TP, E_2, or Dex but not in those given vehicle alone. The order of growth-stimulatory potency for both tumors was TP > E_2 > Dex. Significant differences ($P<0.01$) in tumor size among groups are shown in Fig. 4.

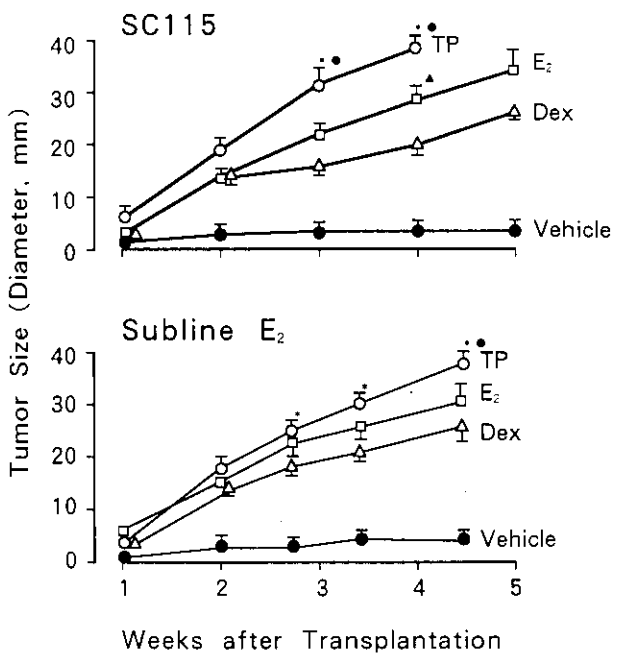

Fig. 4. Stimulatory effects of TP, E_2, or Dex given during the entire experimental period on the growth of SC115 and subline-E_2 tumors in castrated mice. Castrated male mice were transplanted with seed tumors from SC115 or subline E_2 and were given daily injections of TP, E_2, Dex (100 μg/mouse), or vehicle starting from the day of transplantation; subline-E_2 tumors were obtained as described in "Materials and Methods." Values, means ± SE of 6–7 mice; bars, SE. Another trial gave similar results. * $P<0.01$, ● $P<0.01$, and ▲ $P<0.01$, when compared to Dex, E_2 and Dex, respectively.

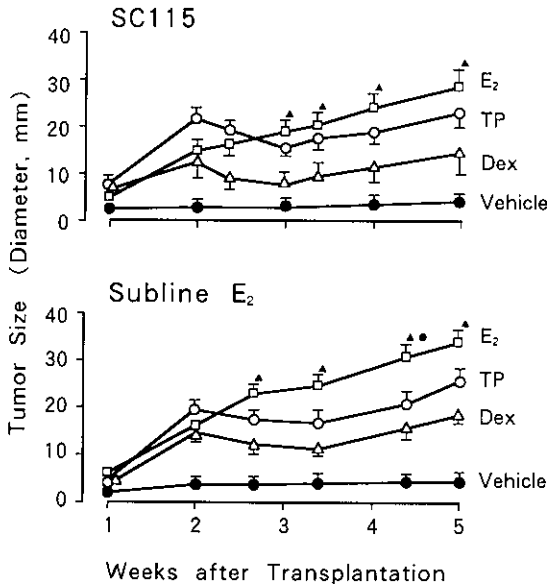

Fig. 5. Stimulatory effects of TP, E_2, or Dex given on days 1–14 after transplantation on the growth of SC115 and subline-E_2 tumors in castrated mice. The treatments were the same as those for Fig. 4, except for a period of steroid injections. ▲ $P<0.01$ and ● $P<0.01$, when compared to Dex and TP, respectively.

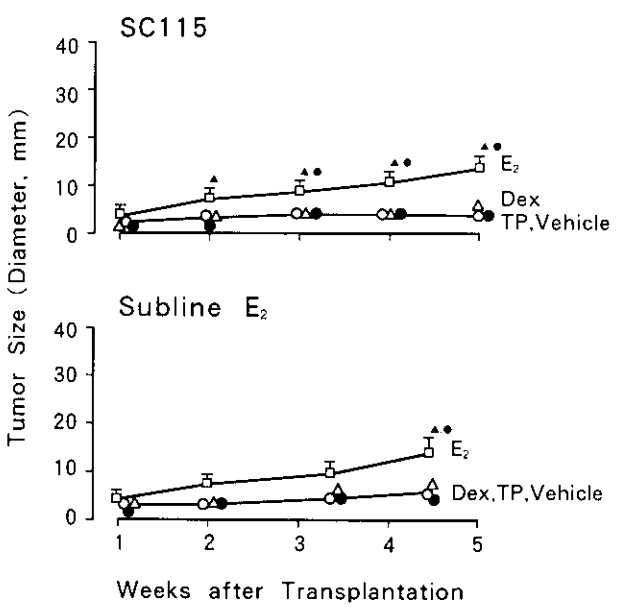

Fig. 6. Effects of TP, E_2, or Dex given on days 1–3 after transplantation on the growth of SC115 and subline-E_2 tumors in castrated mice. The treatments were the same as those for Fig. 4, except for a very short period of steroid injections. ▲ $P<0.01$ and ● $P<0.01$, when compared to Dex and TP, respectively.

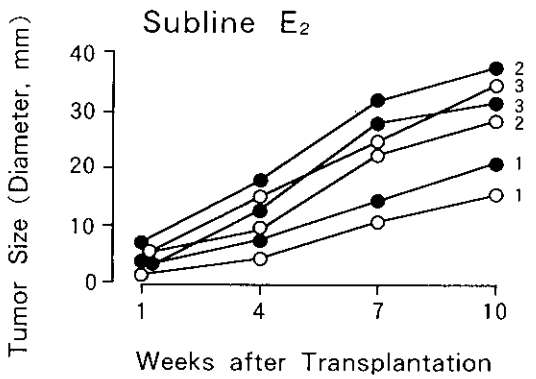

Fig. 7. Loss of androgen responsiveness of subline-E_2 tumors slowly grown in castrated mice for one generation. Seed tumors were obtained from 3 subline-E_2 tumors slowly grown in 3 castrated male mice given only the vehicle. The seed tumors from each mouse (1–3) were transplanted into a group of 6 normal (●) and a group of 6 castrated (○) male mice (1–3); values are the means of 6 mice of each group.

When steroids were given on days 1–14 after transplantation, SC115 and subline-E_2 tumors grown with TP or Dex showed similar temporary regression after steroid withdrawal, followed by growth of tumors in the absence of TP or Dex. However, the tumors grown with E_2 did not show such temporary regression after E_2 withdrawal. Therefore, the order of tumor size at weeks 3 to 5 after transplantation was E_2-treated tumors > TP-treated tumors > Dex-treated tumors ≫ tumors treated with vehicle only; significant differences ($P<0.01$) in tumor size between E_2-treated tumors and Dex- or TP-treated tumors are shown in Fig. 5.

When steroids were given only on days 1–3 after transplantation, TP or Dex had no significant growth-stimulatory effects on SC115 and subline-E_2 tumors. However, E_2 slightly but significantly stimulated the growth of both tumors (Fig. 6).

Seed tumors obtained from slowly grown subline-E_2 tumors in vehicle-treated castrated mice were grafted in normal and castrated males. The growth rate of the seed tumors was similar in normal and castrated males and was clearly slower than that of subline-E_2 tumors grown with TP, E_2, or Dex (Figs. 4 and 7). However, subline-E_2 tumors (maintained by a high dose of E_2) could grow rapidly and significantly only in normal males but not in castrated males (Fig. 8). The loss of response to a high dose (100 μg/mouse/day) of E_2 or Dex of SC115 and subline E_2 tumors was also induced within one passage in steroid-depleted castrated mice (data not shown). The loss of response to androgen, estrogen or glucocorticoid was induced at the same time in steroid-depleted mice.

The growth in response to TP, E_2, or Dex of SC115 tumors was essentially the same as that of subline-E_2 tumors, which had been maintained in castrated mice with a high dose of E_2 for 3 years (30 generations). The present results demonstrate that the preventive effect of high doses of estrogens on the loss of response to androgen, estrogen or glucocorticoid of SC115 tumors is almost the same as the preventive effect of androgens. However, the responsiveness to androgen, estrogen or glucocorticoid of both SC115 and subline-E_2 tumors disappears within one passage in steroid-depleted mice. We have maintained the steroid responsiveness of SC115 tumors for 25 years by using testicular androgens of host mice.

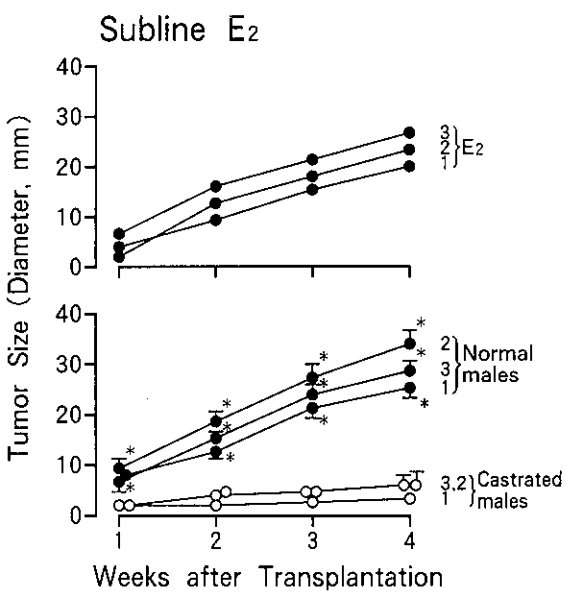

Fig. 8. Androgen responsiveness of subline-E_2 tumors. Seed tumors from each mouse (1–3, top) were transplanted into a group of 6 normal (●) and a group of 6 castrated (○) male mice (1–3, bottom); values are the means±SE of 6 mice of each group. * $P<0.01$, when compared to castrated males.

AR, ER, and GR in cytosols from SC115 and subline-E_2 tumors AR, ER, and GR were demonstrated in cytosols from both SC115 and subline-E_2 tumors, and no significant differences in levels and K_d of AR, ER, and GR were found between SC115 and subline-E_2 tumors (Table I).

DISCUSSION

In cell culture, cloned SC115 cells exhibit a response to androgen in terms of both cell growth[7–14] and production of MMTV-LTR-related RNA[20, 27, 28] but only when these cells are maintained in the presence of androgen. In the prolonged absence of androgen, the cells become unresponsive cells in terms of both parameters.[7, 13, 20, 21, 27, 28] However, it is now evident that SC115 cells are sensitive not only to androgen but also to glucocorticoid both in terms of cell growth[12–15] and MMTV-LTR-related RNA production[27, 28]; androgen and glucocorticoid activities have been shown to be mediated by AR and GR, respectively. The cells unresponsive to one of these two steroids, however, are also unresponsive to the other steroid in terms of these two parameters. In addition, these losses of response are independent of receptor loss, since receptors for both steroids remain not only present but also fully functional.[24] Furthermore, Darbre and King[23, 24] have recently reported that androgen protects via AR against loss of glucocorticoid sensitivity of SC115 cells and *vice versa*. It is now established that binding sites in DNA for different steroid receptors share structural similarities[25] and that the LTR of MMTV is responsive to both androgen and glucocorticoid.[28] These findings can explain the protective effect of androgen or glucocorticoid on MMTV-LTR-related RNA production in SC115 cells. Long-term loss of MMTV RNA in these cells in the absence of androgen and glucocorticoid is ultimately accompanied by increased methylation of MMTV-LTR sequences in the DNA.[20, 27] Therefore, Darbre and King[24] have suggested that the presence of androgen or glucocorticoid can presumably protect against methylation of the glucocorticoid and androgen response element of the responsive genes in SC115 cells,

Table I. AR, ER, and GR in Cytosols from SC115 and Subline-E_2 Tumors

		AR	ER	GR
SC115 tumors	Maximum binding sites (fmol/mg protein)	38 ± 6[a]	16 ± 4	52 ± 3
	K_d (nM)	0.8 ± 0.1	1.2 ± 0.3	3.3 ± 0.5
Subline E_2	Maximum binding sites (fmol/mg protein)	36 ± 5	12 ± 3	48 ± 5
	K_d (nM)	0.6 ± 0.1	1.4 ± 0.2	2.9 ± 0.4

a) Mean±SE of 4 separate determinations.

although the mechanisms of interaction of these steroids at the molecular level remain only speculative, especially with respect to growth.

In the previous studies we have demonstrated that the growth-stimulatory activity of a high dose of E_2 acts on SC115 cells through ER *in vivo*.[16,17] The present findings that the loss of androgen responsiveness of SC115 tumors can be prevented *in vivo* not only by androgen via AR, but also by high doses of estrogen probably via ER, suggest another mechanism for the loss of androgen responsiveness in SC115 cells, because the activity of the ER-estrogen complex can be mediated through the estrogen response element of the response genes.[25]

The loss of androgen responsiveness in the absence of androgen and high doses of estrogen seems to be induced at least in part by selective growth of unresponsive tumor cells produced from SC115 cells. Unresponsive cells become predominant because of the increased death of SC115 cells[29] and slower growth of SC115 than that of unresponsive cells in the absence of androgen or high doses of estrogen. Thus, the loss of androgen responsiveness in SC115 cells occurring in the absence of steroids seems to be induced by a combination of various mechanisms. Androgen-responsive SC115 tumors consist only of medullary cancer cells containing AR, but in the absence of androgen, spindle-shaped cells with or without AR are formed from the medullary cells within the tumors.[1] Since regrown SC115 and subline-E_2 tumors consisted both of medullary cancer cells and clusters of spindle-shaped cells (data not shown), the regrowth of the TP- or Dex-treated tumors following initial response upon treatment removal seems to be due to the appearance of large numbers of autonomous spindle-shaped cancer cells formed from SC115 and subline-E_2 cells in the absence of steroids. However, no reasonable explanation can be given for the lack of regression of the tumors treated with E_2 for 2 weeks upon E_2 removal.

We speculate that the effect of high doses of estrogen *in vivo* is mediated via estrogen-induced growth factor(s) produced in some non-transformed cells, which contain ER with low affinity. However, our data[16] could not convincingly show that the high daily dose of 100 μg of E_2 is acting exclusively through ER *in vivo*. The possibility of an interaction of high doses of E_2 with AR, which was demonstrated by Luthy *et al.*[18] *in vitro*, should be taken into consideration even under *in vivo* conditions.

The growth-stimulatory activities of androgen and high doses of glucocorticoid operate on SC115 cells through AR and GR, respectively, both *in vivo* and in cell culture.[12–15,23,24,30,31] Furthermore, recent studies by us have demonstrated that growth-stimulatory activity of androgen on SC115 cells is mediated through androgen-induced FGF-like peptide in an autocrine mechanism.[30] Androgen incorporated into SC115 cells binds to AR, and FGF-like peptide produced by the AR-androgen complex is secreted from the cells. The secreted FGF-like peptide binds to FGF receptor on SC115 cells and stimulates the growth of the cells.[30] Since anti-FGF antibody IgG markedly and similarly inhibited both testosterone- and Dex-induced growth of SC115 cells,[31] SC115 cells probably produce the FGF-like peptide via GR for their glucocorticoid-induced growth. It is suggested that the *in vivo* growth of SC115 cells in response to androgen and high doses of estrogen or glucocorticoid is induced by different mechanisms, as described before. However, the loss of responsiveness to androgen, estrogen and glucocorticoid occurs at the same time in the absence of these steroids. We suggest that the growth-stimulatory activities of these steroids on SC115 cells may be mediated at least in part through the same pathway, possibly through FGF-like growth factors.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

(Received February 26, 1992/Accepted June 30, 1992)

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