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# GROWTH STIMULATION BY ANDROGENS, GLUCOCORTICOIDS OR FIBROBLAST GROWTH FACTORS AND THE BLOCKING OF THE STIMULATED GROWTH BY ANTIBODY AGAINST BASIC FIBROBLAST GROWTH FACTOR IN PROTEIN-FREE CULTURE OF SHIONOGI CARCINOMA 115 CELLS

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**Summary**—Shionogi carcinoma 115 (SC115) has been accepted for 20 years as an androgen-responsive mouse mammary tumor. We have established an androgen-dependent cloned cell line (SC-3) from a SC115 tumor. In a serum-free medium, testosterone (T) or fibroblast growth factors (FGFs) markedly stimulate the growth of SC-3 cells, and the T-induced growth was shown to be mediated through FGF-like peptide(s) in an autocrine mechanism. Since we used the serum-free culture including 0.1% bovine serum albumin (BSA), a partially serum-containing condition, putative roles of BSA- or serum-borne growth factors in growth stimulation of autocrine production of FGF-like peptide(s) could not be excluded. This paper reports findings performed in a protein-free medium including plating [Ham's F-12:MEM (1:1; v/v)]. In the protein-free culture, the growth of SC-3 cells was significantly stimulated by the addition of $\geqslant 10^{-10}$ M T (up to 20-fold), $\geqslant 10^{-7}$ M dexamethasone (Dex; up to 7-fold) or $\geqslant 1$ ng/ml basic (b) or acidic FGF (up to 10-fold); other various growth factors had no such effects. Furthermore, DNA synthesis of SC-3 cells induced by T, Dex or bFGF was similarly and markedly inhibited by bFGF neutralizing antibody IgG. Therefore, the present findings seem to demonstrate that androgens or high levels of glucocorticoids induce the production and secretion of FGF-like peptide(s) from SC-3 cells for their growth even in the absence of additional support by other factors.

## INTRODUCTION

Androgen-responsive mouse mammary carcinoma, Shionogi carcinoma 115 (SC115), was established in 1964 [1]. It has been generally accepted for 20 years that the growth of SC115 cells is stimulated *in vivo* [2–4] and in cell culture only by androgen [5–9]. This conclusion seems to be reasonable when physiological concentrations of hormones are considered, since SC115 tumor fails to grow in normal adult female mice. However, it has been recently reported that the growth of SC115 cells is also stimulated by high, but not physiological, doses of glucocorticoid both *in vivo* [10] and in cell culture [10–12] and by high, but not physiological, doses of estrogen *in vivo* only [13, 14]. The growth-stimulatory effects of androgen, glucocorticoid and estrogen are mediated through androgen, glucocorticoid and estrogen receptors, respectively [2–14]. We also recently reported that among various growth factors, only fibroblast growth factor (FGF) stimulates the growth of a cloned cell line (SC-3) from SC115 cells [15]. SC-3 cells were obtained from SC115 cells as one of androgen-dependent cloned cell lines [13].

In previous *in vitro* studies by other investigators, serum-supplemented media were used to obtain stimulatory effects of androgens [5–9] on the proliferation of SC115 cells. In order to investigate molecular mechanisms of androgen-induced growth of SC115 cells, culture system in a serum-free medium should be established, since serum contains various growth factors. We have recently established a serum-free culture system [Ham's F-12:Eagle's minimum essential medium (MEM) (1:1; v/v) containing 0.1% bovine serum albumin (BSA)], in which testosterone [10, 13–17] markedly stimulates the

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growth of SC-3 cells. By using the serum-free culture system, we found that the androgen-induced growth of SC115 cells is mediated through androgen-induced autocrine FGF-like peptide(s) [14, 15, 17]. Although we used the serum-free culture, we used methods beginning with cells plated into serum-containing medium. Thereafter, the medium was changed to the serum-free medium containing 0.1% BSA with the addition of mitogens. Therefore, putative roles of serum- or BSA-borne growth factors or regulators in growth stimulation or autocrine production of FGF-like peptide(s) could not be excluded. In order to exclude these possibilities, we have established a protein-free culture including plating [Ham's F-12:MEM (1:1; v/v)]. This paper reports findings that the addition of testosterone, FGFs or high concentrations of dexamethasone significantly stimulates the growth of SC-3 cells in the protein-free medium and that the testosterone-, dexamethasone- or FGF-induced growth is markedly inhibited by anti-basic (b) FGF antibody immunoglobulin G (IgG).

## EXPERIMENTAL

### *Chemicals*

[Methyl-$^3$H]thymidine, steroids, bFGF, acidic(a)FGF, insulin, platelet derived growth factor (PDGF), transforming growth factor (TGF)-$\beta$ and epidermal growth factor (EGF) were obtained as described previously [15, 16]. All other chemicals were of analytical grade.

### *Antibody IgG against bFGF*

IgG fraction of bFGF neutralizing antibody was purchased from R&D systems INC. (Minneapolis, Minn.); the anti-bFGF antibody IgG was prepared in rabbits by injection of highly-purified native bovine brain bFGF. Rabbit IgG used as control was obtained from Sigma Chemical Co. (St Louis, Mo.).

### *Primary cell culture and cloning*

The methods have been described previously [13]. The cloned cell lines were obtained from a SC115 tumor after 20 passages by the limiting dilution method. SC-3 cells (one of androgen-dependent cloned cell lines) were used in the following experiments. The cells were cultured continuously in a maintenance medium composed of MEM containing 2% dextran-coated charcoal (DCC)-treated fetal calf serum (FCS) and $10^{-8}$ M testosterone. Cells were grown in a humidified incubator in 5% $CO_2$ at 37°C.

### *DNA synthesis in cultured cells*

SC-3 cells ($3 \times 10^3$ cells/well) were plated onto a 96-well plate (Coster, Cambrige, Mass.) containing 0.15 ml protein-free medium [Ham's F-12:MEM (1:1; v/v)]. On the following day (day 0), the medium was changed to 0.15 ml of the protein-free medium with or without various concentrations of steroids or growth factors; 3–4 wells were used for each sample. The cells were cultured for 3 days without change of the medium. On day 3, the cells stimulated were pulsed with [$^3$H]thymidine (0.15 $\mu$Ci/0.15 ml per well) for 2 h at 37°C in a humidified incubator and the radioactivity incorporated into the cells was measured; the cells were briefly trypsinized and collected by Cell Harvester LM 101 (Labo Science, Tokyo, Japan). When inhibitory effects of antibody IgG against bFGF on bFGF-, dexamethasone- or testosterone-induced DNA synthesis of SC-3 cells were examined, 500 $\mu$g/ml antibody IgG was added to the protein-free medium containing steroid or bFGF; as control, 500 $\mu$g/ml rabbit IgG was added.

### *Cell growth experiments examined by cell number*

SC-3 cells ($4 \times 10^4$ cells/well) were plated onto a 24-well plate (Coster, Cambrige, Mass.) containing 1 ml of the protein-free medium. On the following day (day 0), the medium was changed

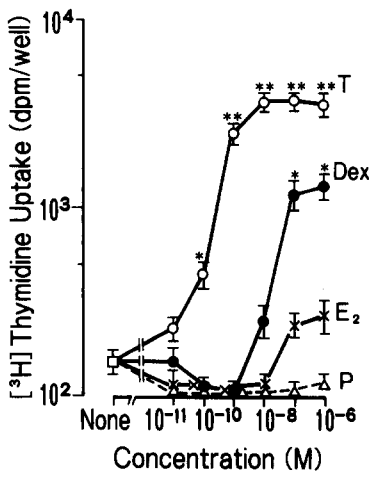


Fig. 1. Effects of $10^{-11}$–$10^{-6}$ M of testosterone (T), dexamethasone (Dex), estradiol-17$\beta$ ($E_2$) or progesterone (P) on [$^3$H]thymidine uptake in SC-3 cells. In a protein-free medium [Ham's F-12:MEM (1:1; v/v)], SC-3 cells were plated, cultured, and examined for [$^3$H]thymidine uptake as described in Experimental. Values, means of 4 determinations; bars, SE; $*P < 0.01$, $**P < 0.001$, when compared to control. The other trial gave similar results.

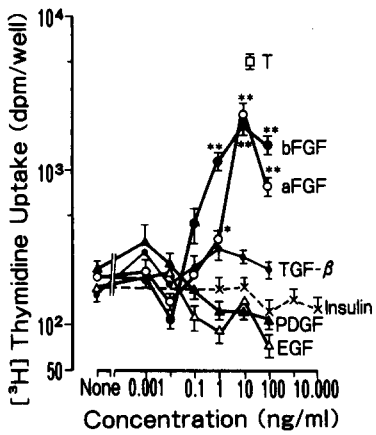


Fig. 2. Effects of various concentrations of various growth factors on [³H]thymidine uptake in SC-3 cells. In the protein-free medium, SC-3 cells were plated, cultured, and examined for [³H]thymidine uptake as described in Experimental. Values, means of 3–4 determinations; bars, SE; $*P < 0.01$, $**P < 0.001$, when compared to control. The other 2 trials gave similar results.

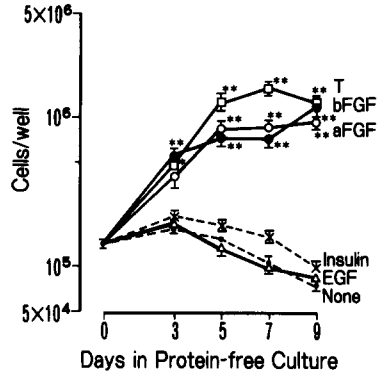


Fig. 3. Effects of testosterone (T; $10^{-8}$ M), bFGF (10 ng/ml, aFGF (10 ng/ml), insulin (10 μg/ml) or EGF (10 ng/ml) on the number of SC-3 cells. In the protein-free medium, SC-3 cells were plated, cultured, and examined for growth as described in Experimental. Values, means of 3–4 determinations; bars, SE; $*P < 0.01$, $**P < 0.001$, when compared to control. The other 2 trials gave similar results.

to 1 ml of the protein-free medium with or without testosterone or growth factors. The medium was changed every other day; 3–4 wells were used for each sample. Cells grown under various conditions were harvested and counted on days 3, 5, 7 and 9 in culture as described previously [13].

## RESULTS

### *Effects of various steroids or growth factors on [³H]thymidine uptake in SC-3 cells in protein-free medium*

The effects of various concentrations of various steroids or growth factors on [³H]thymidine uptake into DNA of SC-3 cells were examined on day 3 in protein-free culture (Figs 1 and 2). Testosterone or dexamethasone stimulated the uptake in SC-3 cells in a concentration-dependent manner. Although the uptake stimulated by testosterone reached a plateau (20-fold) at $10^{-8}$ M, the uptake by dexamethasone reached a plateau (7-fold) at $10^{-6}$ M. Estradiol-17β or progesterone had no significant effects on the uptake in SC-3 cells even at high concentrations, although high concentrations of estradiol-17β slightly increased the uptake (Fig. 1).

Low to high concentrations of EGF, insulin, TGF-β or PDGF showed no significant effect on the [³H]thymidine uptake in SC-3 cells in the protein-free medium. On the other hand, the addition of bFGF or aFGF stimulated the uptake in SC-3 cells in a concentration-dependent manner, and the uptake reached a plateau (up to 10-fold) at the concentration of 10 ng/ml. However, the maximum uptake by FGFs was significantly lower than that induced by testosterone (Fig. 2).

### *Effects of various growth factors or testosterone on the number of SC-3 cells in protein-free medium*

In the protein-free medium without testosterone or FGFs, a significant increase in cell number was not observed during 9 days in culture, even in the presence of EGF (10 ng/ml) or insulin (10 μg/ml). However, the proliferation of SC-3 cells was markedly stimulated by the addition of testosterone ($10^{-8}$ M), bFGF (10 ng/ml) or aFGF (10 ng/ml) until 5 days in culture, resulting in significant increases in cell number on days 3, 5, 7 and 9; up to 10-, 8- and 6-fold increases were obtained by testosterone, bFGF and aFGF, respectively (Fig. 3).

Estimated by cell count as shown in Fig. 3, the stimulation of SC-3 cells was found to be similar order to that estimated by [³H]thymidine incorporation into the DNA of the cells (Figs 1 and 2); highly significant stimulation by testosterone or FGFs and no stimulation by the other growth factors were observed.

### *Inhibitory effects of anti-bFGF antibody IgG on testosterone-, dexamethasone- or bFGF-induced growth of SC-3 cells in protein-free medium*

The effects of bFGF neutralizing antibody IgG on testosterone-, dexamethasone- or bFGF-induced growth of SC-3 cells were examined by measuring [³H]thymidine uptake into the DNA of the cells on day 3 in protein-free culture, since we already found, by using

Table 1. Neutralization of testosterone-, dexamethasone- or bFGF-induced stimulation of [$^3$H]thymidine uptake in SC-3 cells by anti-bFGF antibody IgG. In the protein-free medium, SC-3 cells were plated, cultured in the absence or presence of testosterone, dexamethasone or bFGF plus 500 $\mu$g/ml of antibody IgG or control IgG, and examined for [$^3$H]thymidine uptake as described in Experimental

| Treatment | Absence of IgC | Presence of control IgG | Presence of antibody IgG |
|---|---|---|---|
| | | ([$^3$H]thymidine uptake, dpm/well) | |
| None | 92 ± 11[a] | 107 ± 3 | 124 ± 11 |
| $10^{-8}$ M Testosterone | 8510 ± 480 | 9170 ± 910 | 3120 ± 270** |
| $10^{-6}$ M Dexamethasone | 1780 ± 201 | 1850 ± 104 | 771 ± 48** |
| 1 ng/ml bFGF | 4970 ± 251 | 4890 ± 326 | 1130 ± 143** |

[a]Mean ± SE of 3 determinations. **$P < 0.001$, when compared to the presence of control IgG. Other trials, 3 for testosterone or bFGF and 1 for dexamethasone, gave similar results.

the serum-free medium containing 0.1% BSA, that SC-3 cells produce FGF-like peptide(s) for their testosterone-induced growth [15, 17]. In preliminary experiments, 400–600 $\mu$g/ml of bFGF neutralizing antibody IgG (R&D Systems Inc.) induced the greatest inhibition of testosterone ($10^{-8}$ M)-induced [$^3$H]thymidine uptake in SC-3 cells, whereas 400–600 $\mu$g/ml of control IgG showed no significant effect. Therefore, 500 $\mu$g/ml of anti-bFGF antibody IgG was used to examine inhibitory effects of the antibody IgG on testosterone-, dexamethasone- or bFGF-induced growth of SC-3 cells; 500 $\mu$g/ml of control IgG was used as control. As shown in Table 1 and Fig. 4, [$^3$H]thymidine uptake into DNA of the cells induced by 1 ng/ml bFGF, $10^{-6}$ M dexamethasone or $10^{-8}$ M testosterone was markedly and significantly inhibited by the addition of 500 $\mu$g/ml of anti-bFGF antibody IgG. Figure 4 shows that 67, 63 and 79% inhibition of the testosterone, dexamethasone and bFGF effect was attained, respectively.

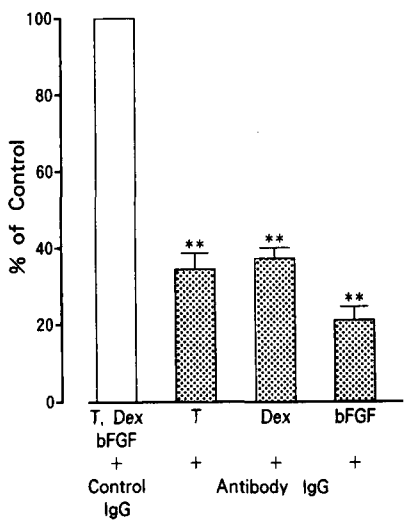


Fig. 4. Neutralization of testosterone (T; $10^{-8}$ M)-, dexamethasone (Dex; $10^{-6}$ M)- or bFGF (1 ng/ml)-induced stimulation of [$^3$H]thymidine uptake in SC-3 cells by anti-bFGF antibody IgG. In the protein-free medium, SC-3 cells were treated as shown in Table 1. The control value (100%) represents the amount of [$^3$H]thymidine uptake produced by testosterone, dexamethasone or bFGF plus 500 $\mu$g/ml control IgG. The neutralization obtained in the presence of testosterone, dexamethasone or bFGF plus 500 $\mu$g/ml antibody IgG is shown as a percentage of this control value. Values, means of 3 determinations; bars, SE; **$P < 0.001$, when compared to control (testosterone, dexamethasone or bFGF plus control IgG). Other trials, 3 for testosterone or bFGF and 1 for dexamethasone gave similar results.

*Morphology of SC-3 cells in protein-free medium*

SC-3 cells grown in unsupplemented protein-free medium showed an epithelial appearance. The SC-3 cells changed to a fibroblast-like appearance only in the presence of $\geqslant 10^{-9}$ M testosterone, $10^{-6}$ M dexamethasone or $\geqslant 1$ ng/ml FGFs, which significantly stimulated the growth of SC-3 cells (Fig. 5). Various concentrations of other steroids and growth factors, which did not stimulate the growth, had no effect on the morphology of SC-3 cells.

## DISCUSSION

Although other investigators used serum-supplemented media to obtain stimulatory effects of androgens [5–9] or high concentrations of glucocorticoids [11, 12] on the growth of SC115 cells, we have already reported such growth-stimulatory effects on SC-3 cells derived from SC115 in a serum-free medium [10, 13–18]; estradiol-17$\beta$ or progesterone had no significant effect [13–15]. Furthermore, we found a significant growth-stimulatory activity of FGFs on SC-3 cells; other various growth factors such as EGF, TGF-$\alpha$, insulin, insulin-like growth factor (IGF)-I, PDGF or TGF-$\beta$ had no significant growth-stimulatory effects [15]. Furthermore, SC-3 cells changed morphology only in the presence of androgens, FGFs or dexamethasone [13, 15, 17]. These previous findings [10, 13–18] we obtained by using the serum-free medium are similar to those obtained in the present study using a protein-free medium (Figs 1–3 and 5).

Recent studies have shown that growth-stimulatory effects of sex steroids on cell proliferation are mediated by specific polypeptide growth factor(s) [19]. In estrogen-responsive

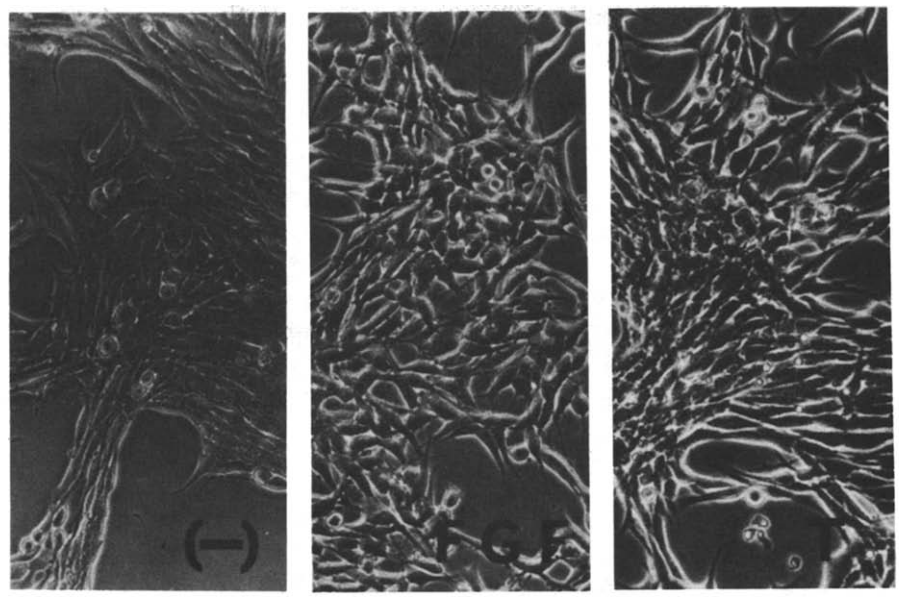


Fig. 5. Morphological alteration in SC-3 cell shape induced by testosterone (T; $10^{-8}$ M) or bFGF (10 ng/ml). In the protein-free medium, SC-3 cells were plated and cultured in the absence and presence of testosterone or bFGF. On day 4 in culture, cells were photographed through contract optics ×150.

cancer cells, Lippman *et al.* [20] have suggested that all stimulatory effects of estrogen on the growth of human breast cancer cells such as MCF-7 are mediated through 30 kDa TGF-α- or IGF-I-like factor in an autocrine mechanism. Since Lippman *et al.* [20] have used serum-supplemented medium including partially serum-free conditions, Karey and Sirbasku [21] have recently established a culture of MCF-7 cells using a serum-free medium containing human transferrin (10 μg/ml) and BSA (200 μg/ml). They [21] found that the concentration of IGF-I, which is the most active growth-stimulating factor examined, is not increased by estradiol-17β treatment and the effect of estradiol-17β on the growth of MCF-7 cells is minimal. These findings in the serum-free medium opened possibilities that additional serum-borne hormones, growth factors or regulators are probably required for IGF-I (TGF-α) autocrine induction by estradiol-17β. Even in the study of Karey and Sirbasku [21], however, possible roles of some factors contaminated in BSA in autocrine growth factor induction could not be excluded. Furthermore, Arteaga *et al.* [22] also reported that antibodies that block the EGF/TGF-α receptor, inhibit TGF-α-induced but not estrogen-induced growth of human breast cancer cells. In androgen-responsive cancer cells, a preliminary finding by others appeared recently [23]; serum-supplemented medium conditioned by testosterone-treated SC115 cells stimulated the growth of L929 mouse fibroblasts, but the nature of the testosterone-induced factor(s) was not elucidated. These previous findings by others indicate that the establishment of a protein-free culture is desirable in order to investigate molecular mechanisms of sex steroid-induced growth in relation to growth factor(s).

By using the serum-free culture system which contains 0.1% BSA, we have already reported the following findings. Conditioned medium containing testosterone-induced secretory factor(s) from SC-3 cells markedly stimulated the proliferation of SC-3 cells in the absence of testosterone activity [14, 17]. The testosterone-induced factor(s) was adsorbed in a heparin–Sepharose column, which was eluted with 1.1 M NaCl [17]. The partially purified factor(s), which was found to be included in heparin-binding growth factor family [24–26], was 40 kDa under the nondenaturing conditions, and bound to FGF receptors on SC-3 cells with high affinity [27]. Androgen antagonists such as cyproterone acetate markedly inhibited the growth of SC-3 cells induced by testosterone but not at all by the testosterone-induced FGF-like factor(s), suggesting that androgen-receptor complexes induce production of the FGF-like factor(s) in SC-3 cells [14, 17, 18]. Furthermore, DNA synthesis of SC-3 cells induced by testosterone or bFGF was similarly and markedly inhibited by bFGF neutralizing antibody IgG [18]. These

findings already observed by us using the serum-free culture seem to demonstrate that growth-stimulatory effects of testosterone on SC-3 cells are mediated through testosterone-induced autocrine FGF-like peptide(s). However, possible roles of BSA- or serum-borne growth factors or regulators in growth stimulation or autocrine production of FGF-like peptide(s) could not be excluded in these studies using the serum-free culture including BSA, partially serum-containing condition. These possible roles seem to be excluded by the present study using the protein-free culture beginning from plating, since findings similar to those obtained in the serum-free culture [14, 15, 17, 18] were obtained even in the present protein-free culture. As shown in Figs 1 and 2, testosterone was more effective than FGFs in stimulating [$^3$H]thymidine uptake in SC-3 cells. It is suggested that FGF-like peptide (40 kDa) induced from SC-3 cells by testosterone is more effective than FGFs (20 kDa), since the FGF-like peptide was similar to, or more effective than, testosterone in growth stimulation of SC-3 cells [17, 27]. Furthermore, FGF-stimulated growth of SC-3 cells could not be enhanced at all by all the other growth factors examined (manuscript in preparation). The present findings shown in Fig. 4 also suggest that SC115 (SC-3) cells produce FGF-like peptide for their glucocorticoid-induced growth, since anti-bFGF antibody IgG clearly inhibited the glucocorticoid-induced growth.

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