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NON-CLASSICAL ANDROGENIC ACTIONS OF RU38486 IN ANDROGEN-RESPONSIVE SHIONOGI CARCINOMA 115 CELLS IN SERUM-FREE CULTURE

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Summary—Antiglucocorticoid and antiprogestin RU38486 (RU486) stimulated the growth of highly androgen- and moderately glucocorticoid-sensitive SC-3 cells (a cloned cell line from Shionogi mouse mammary carcinoma 115) in a dose-dependent manner. A maximal 8-fold stimulation of growth by RU486 has been observed at 10^{-7} M in a serum-free medium and its potency has been found to be almost the same as that of dexamethasone (Dex). The growth rate of SC-3 cells treated by triamcinolone acetonide (TA) or Dex combined with RU486 at 10^{-9}–10^{-7} M was enhanced compared to cells treated by TA or Dex alone, indicating that RU486 had additive rather than antagonistic effects. Our previous study revealed that RU486 could compete with the specific uptake of [^{3}H]testosterone in intact SC-3 cells at relatively low affinity and the present study showed that the stimulatory effect of RU486 on the growth of SC-3 cells was significantly inhibited by pure antiandrogen flutamine and that half-maximal inhibition by flutamine was achieved at 10^{-6} M. Moreover, we demonstrated that the conditioned medium from RU486-stimulated SC-3 cells contained growth-promoting activity which caused a 3.5-fold increase in DNA synthesis by SC-3 cells in the absence of RU486 and which was abolished by treatment with heparin-Sepharose. These results indicate that RU486-induced growth of SC-3 cells may be expressed as an androgenic activity through androgen receptor and mediated by a heparin-binding growth factor.

INTRODUCTION

The growth of highly androgen- and moderately glucocorticoid-sensitive SC-3 cells (a cloned cell line from the Shionogi mouse mammary tumor SC115) has provided a useful model for the study of the growth-modulating action of steroid hormones, their antagonists and growth factors [1–5]. In a serum-free medium [Ham's F-12: Eagle's minimum essential medium (MEM) (1:1, v/v) containing 0.1% bovine serum albumin (BSA)], we found that physiological doses of androgens, high doses of glucocorticoids and 1–10 nM fibroblast growth factors (FGFs) significantly stimulated the growth of SC-3 cells through their respective receptors. Furthermore, DNA synthesis in SC-3 cells induced by androgens or glucocorticoids was significantly inhibited by the addition of bFGF neutralizing IgG antibody to the medium [3–5]. It was also demonstrated that SC-3 cells had typical FGF receptors with an apparent molecular mass of 130,000, whose labeling was inhibited when basic FGF, acidic FGF or highly purified androgen-induced growth factor was present in excess [6]. These results suggest that the growth-stimulatory activity of androgen or glucocorticoid on SC-3 cells is mediated by an FGF-like peptide(s) through an autocrine mechanism. Since RU38486 (RU486) (a strong antagonist against glucocorticoid and progestin) showed an antiproliferative effect in some tumors [7, 8], its effect on the growth of SC-3 cells was also studied. In our previous study, RU486 unexpectedly showed growth-stimulatory activity similar to that of dexamethasone (Dex) [5]. In order to further understand the growth stimulating potency of RU486 and its molecular mechanism in SC-3 cells, we used the serum-free culture system to investigate in more detail the effect of RU486 on SC-3 cell growth and compared this effect with those of testosterone (T) and potent glucocorticoid agonists on the same parameters.

MATERIALS AND METHODS

Chemicals

[Methyl-^{3}H]thymidine (40 Ci/mmol) was obtained from Radiochemical Center, Amersham

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(Bucks., U.K.). Testosterone (T), triamcinolone acetonide (TA), Dex, flutamine and BSA (essential fatty acid-free) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). RU486 was supplied by Roussel-Uclaf (Paris, France). Heparin-Sepharose CL-6B and Sepharose CL-6B were from Pharmacia (Piscataway, NJ, U.S.A.). The other chemicals were of analytical grade.

Primary cell culture and cloning

The methods used in these studies were as described previously [9]. Cloned cell lines were obtained from a SC115 tumor after 20 passages by the limiting dilution method and SC-3 cells (one of androgen-responsive cloned cell lines) were used in the experiments reported here. The cells were cultured continuously in a maintenance medium composed of MEM containing 2% dextran-coated charcoal-treated fetal calf serum (DCC-FCS) and 10^{-8} M T. Cells were grown in a humidified incubator in 5% CO_2 at 37°C.

DNA synthesis in cultured cells

SC-3 cells were plated onto a 96-well plate (Coster, Cambridge, MA, U.S.A.) at 3×10^3 cells/well in 0.15 ml of MEM with 2% DCC-FCS. On the following day (day 0), the medium was replaced by 0.15 ml of serum-free medium [Ham's F-12: MEM (1:1 v/v) containing 0.1% BSA] with or without test compounds. Three wells were used for each sample. Steroids dissolved in ethanol solution was added to the medium, in which the final concentration of ethanol was 0.1%. Control wells also contained 0.1% ethanol. The cells were then cultured for 3 days without changing medium. On day 3, the cells stimulated were pulsed with [^{3}H]thymidine (0.15 μCi/0.15 ml per well) for 2 h at 37°C in a humidified incubator and the incorporation of radioactivity into the cells, briefly trypsinized and collected by cell harvester LM 101 (Labo Science, Tokyo, Japan), was measured. Three wells were used for each sample. When the effect of the conditioned medium (CM) from T-, Dex- or RU486-stimulated SC-3 cells (T-, Dex- or RU486-CM) was examined, the medium in each well was replaced by 0.15 ml of CMs on day 0 and day 2. CM from non-stimulated cells (Non-CM) was used as control.

Cell growth experiment examined by measuring cell numbers

SC-3 cells were plated onto a 24-well plate (2×10^4 cells/well) containing 1 ml of MEM with 2% DCC-FCS. On the following day (day 0), the medium was replaced by 1 ml of serum-free medium with or without test compounds. After that the media were changed every other day and three wells were used for each sample. Cells grown under the various conditions were harvested and counted at indicated times, as described previously [9].

Preparation of T-, Dex- and RU486-CMs

SC-3 cells (1.0×10^6 cells/100 mm-dish) were plated in MEM containing 2% DCC-FCS with or without 10^{-8} M T, 10^{-7} M Dex or 10^{-7} M RU486. On the following day (day 0), the media were substituted with serum-free media with or without T, Dex or RU486 after the cells were washed twice with MEM. On day 3, the cultures were washed with MEM and subjected to a second washing after incubation with the same medium at 37°C for 2 h. The media were then replaced by serum-free medium without T, Dex or RU486 which were removed 24 h later to obtain T-, Dex- and RU486-CMs. The cells plated and cultured in medium without steroids were used for preparing Non-CM. The CMs and Non-CM were centrifuged at 1000 *g* for 5 min to remove cell debris before they were passed through a 0.22 μm filter and then stored at −20°C for use.

Treating CMs with heparin-Sepharose

The 50 ml of collected CMs were added to heparin-Sepharose or Sepharose (gel volume: 0.25 ml) which had been preequilibrated with 10 mM Tris–HCl buffer (pH 7.0 at 20°C) containing 0.1% NaCl and 0.2% (w/v) CHAPS. The tubes were inverted and reverted three times before they were immersed in an ice bath and kept there for 5 min. This procedure was repeated three times. Suspensions were centrifuged at 1000 *g* for 5 min and the supernatants were filtered under sterile conditions and stored at −20°C.

RESULTS

Growth-stimulatory effects of glucocorticoids and RU486 on SC-3 cells in serum-free medium

The effects of various concentrations of potent glucocorticoid agonists, TA, Dex, and of RU486 on [^{3}H]thymidine uptake into DNA in SC-3 cells were studied in the serum-free medium [Fig. 1(A)]. TA and Dex significantly stimulated [^{3}H]thymidine uptake in a dose-

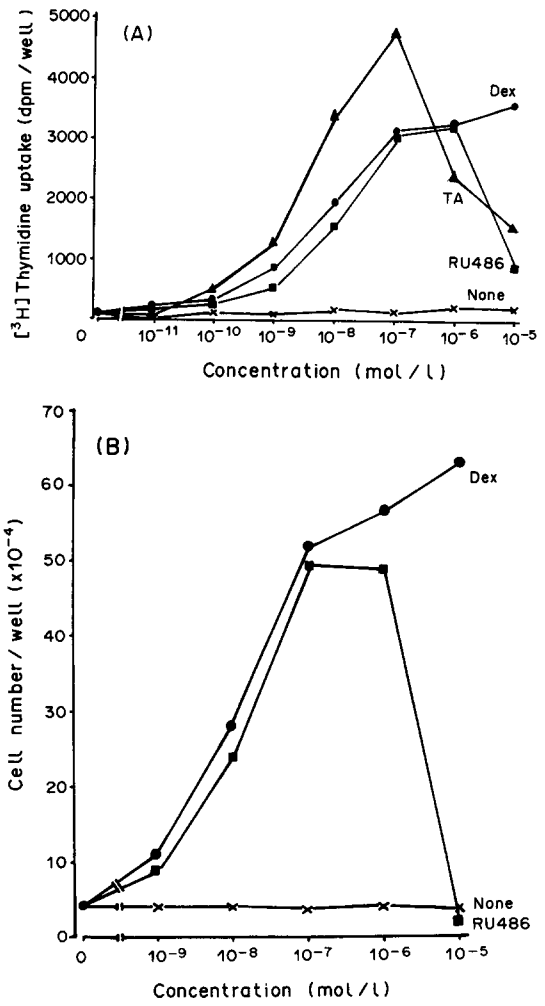

Fig. 1. Stimulatory effects of increasing concentrations of glucocorticoids or RU486 on DNA synthesis and cell proliferation of SC-3 cells. SC-3 cells were cultured in the absence or presence of various concentrations of glucocorticoid(s) or RU486. [^{3}H]thymidine uptake on day 3 (A) and cell number on day 12 (B) were measured as described in Materials and Methods. Values are means of 3 determinations; the other 3 separate trials also showed similar results.

dependent manner, with a maximal effect at 10^{-7} M and 10^{-6} M, respectively. Unexpectedly, RU486 also displayed the dose-dependent growth-stimulatory activity over the range of 10^{-9}–10^{-6} M. Growth-stimulatory effect achieved by 10^{-7} M RU486 was about 66% and 95% of the values achieved by TA and Dex, respectively, at the same concentrations. Further elevation of RU486 to 10^{-5} M resulted in a significantly decreased DNA synthesis. Cell numbers determined on day 12 showed the same result [Fig. 1(B)]. The reason for the growth-inhibition elicited by high concentrations of RU486 is unclear, perhaps being due to a non-specific cytotoxic effect of the agent.

The stimulatory effects of 10^{-8} M T, 10^{-7} M Dex and 10^{-7} M RU486 on the number of SC-3 cells were examined next (Fig. 2). After 15 days of culture in serum-free medium, proliferation rate was significantly increased by T (up to about 130-fold), Dex (up to 8.5-fold) and RU486 (up to 8-fold). The stimulatory effects of Dex and RU486 were found to be of an order similar to those estimated by [^{3}H]thymidine incorporation into the DNA of cells (Fig. 1). These results clearly demonstrated that RU486 is as potent as Dex at 10^{-7}–10^{-6} M concentrations as a stimulator of cell growth.

Influence of the antiandrogen flutamine on growth-promoting effects of T, glucocorticoids and RU486 in SC-3 cells

Our previous studies [3] have indicated that SC-3 cells contain androgen (AR) and glucocorticoid receptor (GR). RU486 could not only bind to GR with higher affinity than that of Dex, but also showed competitive binding to AR, with relatively low affinity. Since the stimulatory effect of RU486 on growth of SC-3 cells was inhibited by antiandrogen cyproterone acetate (CA), it is suggested that the growth induced by RU486 may be mediated by AR. In

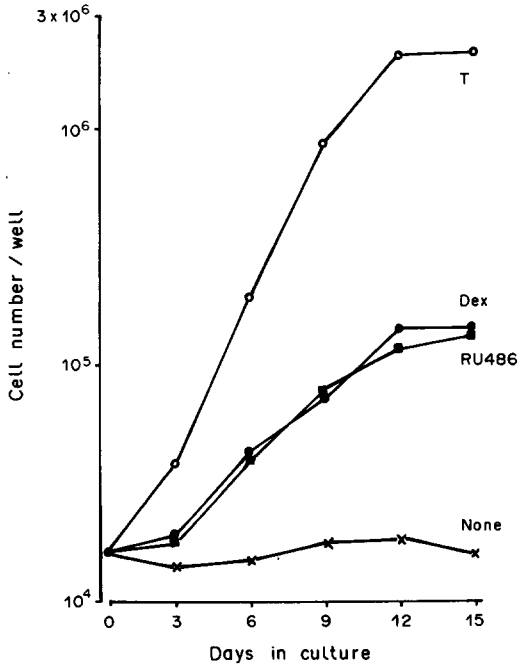

Fig. 2. Stimulatory effects of T, Dex or RU486 on the number of SC-3 cells. SC-3 cells were cultured in the absence or presence of 10^{-8} M T, 10^{-7} M Dex or 10^{-7} M RU486 and the number of cells per well was counted on the indicated days as described in Materials and Methods. Values are means of 3 determinations; the other separate trial also showed a similar result.

order to confirm the results reported previously, we used flutamine, a pure non-steroidal anti-androgen, to study its effect on stimulation of cell growth induced by T, glucocorticoid and RU486. As clearly illustrated in Fig. 3, flutamine alone had no effect on DNA synthesis in SC-3 cells but, when simultaneously added to media with 10^{-8} M T, 10^{-7} M TA, 10^{-7} M Dex or 10^{-7} M RU486, flutamine inhibited the T- and RU468-induced DNA synthesis in a dose-dependent fashion. The maximal effect of flutamine was achieved at 10^{-5} M, at which it almost caused a complete inhibition of the stimulatory effects of T and RU486, but had no significant effects on the mitogenic activity of TA or Dex. Such data strongly support an essential role of the AR as mediator of the effect of RU486 on cell proliferation.

Growth-promoting activity of RU486-CM

In order to investigate if the growth-promoting effect of RU486 is mediated by some growth factor(s), the growth-promoting activity in the RU486 stimulated CM was examined. To characterize the growth promoting factor in RU486-CM, it is critically important to cancel the RU486 activity present in the CM. DCC treatment has been widely used to remove steroid hormone; however, it has been known to absorb not only hormonal steroids but also some growth-regulating factors [10]. Another method commonly used is to add to CM an steroid antagonist. Nevertheless, these additions would increase the complexity of the experiment. The simple method used here is based on the supposition that SC-3 cells stimulated by RU486 could continue to secrete great amounts of growth factor [5] into medium even during the 24 h period in the absence of RU486. In this experiment, T- and Dex-CMs were used as a positive control because we have shown that the growth-promoting effects of T and Dex are mediated by FGF-like growth factor(s). As shown in Fig. 4, T-, Dex- or RU486-CM significantly stimulated the uptake of [^{3}H]thymidine into SC-3 cells (up to 8-, 3- and 3.5-fold above control, respectively). Non-CM was also found to have growth-promoting activity. However, its potency was lower than that of CMs. Treatment

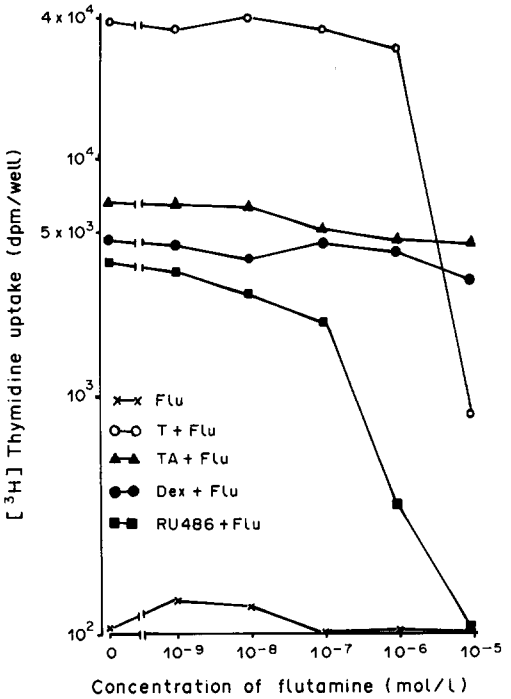

Fig. 3. Inhibitory effect of increasing concentrations of flutamine (Flu) on a fixed concentration of T, glucocorticoids- or RU486-induced DNA synthesis in SC-3 cells. SC-3 cells were cultured in the absence or presence of increasing concentrations of flutamine with or without 10^{-8} M T, 10^{-7} M TA, 10^{-7} M Dex, or 10^{-7} M RU486. The [^{3}H]-thymidine uptake in the cells was estimated as described in Materials and Methods. Values are means of 3 determinations; the other 2 separate trials also showed similar results.

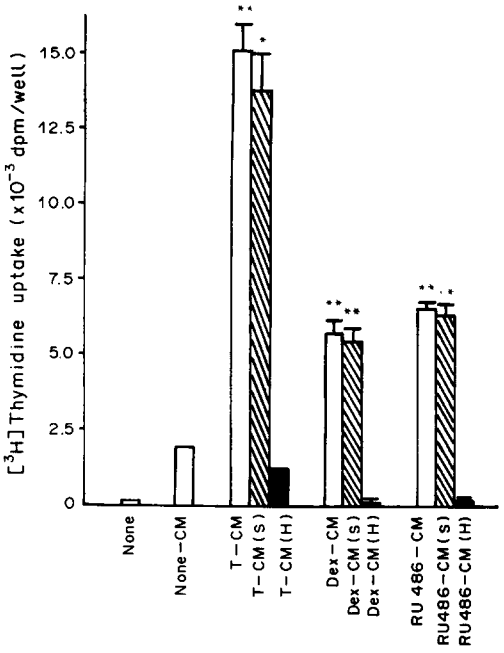

Fig. 4. Effect of T-, Dex- or RU486-CM with or without treating with heparin-Sepharose or sepharose on DNA synthesis in SC-3 cells. SC-3 cells were plated onto 96-well plate (4×10^3). On the following day (day 0), the medium was changed to 0.15 ml of Non-CM as well as T-, Dex- or RU486-CM treated with or without heparin-Sepharose [CM (H)] or Sepharose [CM (S)]. CMs were changed on day 2. On day 3, [^{3}H]thymidine uptake into SC-3 cells was estimated. Values are means of 3 determinations; Bars = SE. The other separate trial also showed a similar result.

of CMs with Sepharose had no significant effect but when CMs were treated with heparin-Sepharose, >95% of the growth-promoting activity of CMs was abolished. The result indicated that similar to T and Dex, stimulation of SC-3 cells by RU486 resulted in a secretion of autocrine growth factor(s) which could be adsorbed by heparin-Sepharose and display characteristics similar to those of FGFs.

The interaction of glucocorticoids and RU486 on the growth of SC-3 cells

It then became of interest to study the interaction between glucocorticoids and RU486 as stimulators of cell growth. As can be seen in Fig. 5(A) and (B), in contrast to an antagonistic effect, RU486 was found to further accelerate the TA- and Dex-induced cell proliferation in a dose-dependent manner at moderate concentration (10^{-9}–10^{-7} M). However, at a higher concentration (10^{-6} M), RU486 decreased cell proliferation rate to an extent not lower than that as induced by TA or Dex alone. We did not conduct this experiment with RU486 at 10^{-5} M because of its growth-inhibitory effect on SC-3 cells at this concentration (Fig. 1). These results indicated that RU486 not only had the ability to stimulate growth of SC-3 cells alone, but also showed additive effects on TA- or Dex-enhanced cell proliferation at 10^{-9}–10^{-7} M.

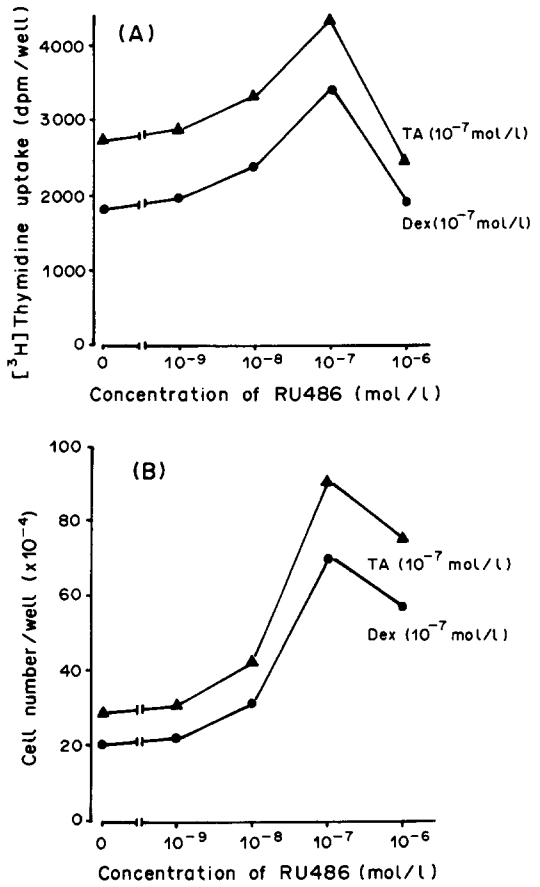

Fig. 5. Effects of increasing concentrations of RU486 on a fixed concentration of glucocorticoid-induced DNA synthesis and cell proliferation rate in SC-3 cells. SC-3 cells were cultured in the absence or presence of increasing concentrations of RU486 with or without 10^{-7} M TA or 10^{-7} M Dex. [^{3}H]thymidine uptake on day 3 (A) or cell number on day 12 (B) were measured as described in Materials and Methods. Values are means of 3 determinations; the other 2 separate trials also showed similar results.

DISCUSSION

The present study has clearly demonstrated that RU486 has a significant growth-stimulatory effect on androgen- and glucocorticoid-sensitive SC-3 cells in a serum-free medium. The stimulatory ability is similar to that of Dex. These results are unexpected, since the vast majority of studies have reported a contrast action of RU486 to that of glucocorticoid in various normal tissues or tumor cell lines *in vivo* and *in vitro* [11–15]. Nevertheless, this non-classical stimulatory effect of RU486 was uniquely observed in SC115 cells. SC-3 cells are an androgen- and glucocorticoid-sensitive cell line and contain both AR and GR. Furthermore, as we know, RU486 not only binds to GR but also binds to progesterone receptor (PR) with high affinity. This would raise an interesting question, i.e. what mediates the action of RU486, GR, AR or PR? Since SC115 tumor and SC-3 cells contained no PR [4, 16], we can exclude the possibility that RU486 could act through PR. Our previous and present results revealed that RU486 could competitively bind to AR, although with relatively low affinity, and that a stimulatory effect of RU486 on the growth of SC-3 cells was significantly blocked not only by steroidal antiandrogen, CA, but also by the pure non-steroidal antiandrogen, flutamine. It is suggested that RU486 exhibits some androgenic effects on SC-3 cells. In other words, the growth-stimulatory effect of RU486 may be via a AR-mediated mechanism. Because SC-3 cells is a highly androgen-sensitive cell clone, the growth-stimulatory ability of androgen is much higher than that of Dex in serum-free medium. T could increase the proliferation of SC-3 cells to 130-fold at 10^{-8} M whereas Dex could only increase it to 8.5-fold at 10^{-7} M (Fig. 2). This quantitative difference between androgen- and glucocorticoid-dependent growths of

SC-3 cells indicates that the androgen–AR complex is more active than the glucocorticoid–GR complex. This could probably explain the present observation that RU486 showed a significant stimulatory effect on the growth of SC-3 cells in spite of its low affinity for AR.

The growth-promoting action of RU468 have also been noted by Labrie *et al.* [11] in another clone (SEM-1) of the SC115 cell line, indicating that this is a cell type-specific event. But in their case, while causing 80% inhibition of the action of TA in those cells, RU486 only showed a small growth-stimulatory effect at its maximal concentration, approximating 30% of the value achieved by a similar concentration of TA. The different response patterns in those two cell clones indicates that the original Shionogi tumors may contain cells having a wide range of sensitivities to androgens. The same group also reported that 17β-estradiol stimulates the growth of SEM-1 cells mediated by the AR [18].

By using a serum-free culture system, we have already reported that the stimulation of SC-3 cells by androgen resulted in secretion of autocrine growth factor(s) with a M_r of 40,000 under denaturing conditions [3, 6]. This androgen-induced growth factor(s) was adsorbed in heparin affinity column and was eluted at 1.1 M NaCl. We also found that Dex induced in SC-3 cells the synthesis of secretory proteins identical to those induced by T via GR [19]. Furthermore, antiFGF antibody IgG clearly inhibited both androgen- and glucocorticoid-induced growth of SC-3 cells [5, 17]. These findings suggest that the effects of androgens and glucocorticoids might have been mediated in a FGF-like peptide(s) through an autocrine mechanism. Actually, only basic and acidic bFGFs have been observed to stimulate the growth of SC-3 cells among various growth factors examined [4, 17]. The present study presents preliminary findings, indicating that CM prepared from RU486-stimulated SC-3 cells markedly promoting DNA synthesis in the absence of RU486 and that the growth-promoting activity was abolished by the treatment with heparin-Sepharose. In addition, the morphology of SC-3 cells showed the same alteration from epithelial shape to fibroblast-like appearance in the medium supplemented by RU486 and Dex (data not shown). These results indicate that similar to the case with T and Dex, RU486-induced enhancement of SC-3 cell growth is also mediated through an induction of FGF-like growth factor which act on SC-3 cells as an autocrine growth factor.

In addition to demonstrating a growth-promoting action of RU486 itself, the present finding demonstrated for the first time that RU486 failed to display a suppressive effect even at macromolecular concentrations. On the contrary, it demonstrated an additive effect on glucocorticoid-induced growth of SC-3 cells with a maximal effect at 10^{-7} M. However, a higher dose of RU486 caused a decreased proliferation. The molecular mechanism of the paradoxical effect of RU486 on glucocorticoid-induced cell proliferation is largely unclear. Although this experiment did not allow us to exclude the possibility that RU486 could enhance the cell proliferation stimulated by glucocorticoids via increasing GR number, we prefer to believe that event might have occurred at post-receptor level. The recent results of cDNA cloning and the comparison of the deduced amino acid sequence of different steroid hormone receptors reveal that all steroid receptors share a short and well conserved cysteine-rich central domain (DNA-binding region) [20]. This homology in DNA-binding domain among steroid receptors has led some investigators to propose the possibility that several steroid hormone receptors could interact with a common hormone responsive element (HRE) to regulate specific gene expression. Some reports have supported this hypothesis [21–24]. Barbre *et al.* [25] have reported that in SC115 cells, androgen as well as glucocorticoid can act directly on the long terminal repeat (LTR) of mouse mammary tumor virus (MMTV) to increase the gene expression through AR and GR, respectively. By using another kind of breast cancer cell (T-47-D), they have also shown that the multihormone regulation of MMTV-LTR occurs through the same HRE site and by the enhancer-type mechanism [26]. Our previous and present results have revealed that androgen, glucocorticoid and RU486 are all able to stimulate SC-3 cells to produce the FGF-like growth factor(s) for their growth-promoting effect, which suggests that the androgen–AR, RU486–AR as well as glucocorticoid–GR complexes could act on a specific gene whose products are directly linked to cell proliferation through the same HRE in SC-3 cells, but the transcriptional interaction between the RU486–AR and Dex–GR complexes remains to be investigated.

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