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An Essential Role of Androgen-induced Growth Factor in Glucocorticoid-dependent Autocrine Loop in Shionogi Carcinoma 115 Cells

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Androgen-induced growth factor (AIGF) is essential for the androgen-induced autocrine growth of a mouse mammary Shionogi carcinoma cell line (SC-3 cells). Because glucocorticoid and estrogen have been observed to weakly stimulate DNA synthesis in SC-3 cells, the expression of AIGF mRNA after stimulation with various concentrations of androgen, glucocorticoid, or estrogen was examined by Northern blot analysis. Testosterone, dexamethasone, and estradiol-17β (E_2) induced AIGF mRNA expression, although the maximum AIGF mRNA expression levels induced by dexamethasone or E_2 were lower than that by testosterone. Yet, diethylstilbestrol showed no induction, suggesting that the effect of E_2 could be mediated through the androgen receptor. The induction levels of AIGF mRNA by each steroid hormone were correlated positively with hormone-induced DNA synthesis. In addition, the DNA synthesis induced by each steroid hormone was almost completely inhibited by AIGF antisense oligonucleotides, indicating that AIGF is an obligatory component in not only the androgen- but also the glucocorticoid-inducible autocrine loop in SC-3 cells.

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INTRODUCTION

The androgen-induced growth of SC-3 cells (a cloned cell line from mouse mammary carcinoma SC115) has been proved to be mediated through the induction of a growth factor [1–3]. Our recent study on the cloning of this growth factor, termed androgen-induced growth factor (AIGF), has revealed that AIGF belongs to the fibroblast growth factor (FGF) family of proteins [4]. Because AIGF mRNA is induced markedly by testosterone and AIGF alone exerts an androgen-like growth stimulatory effects on SC-3 cells, AIGF can be considered to be an obligatory component in the androgen-inducible autocrine loop [5].

We and other investigators have shown that the growth of SC115-derived cells was also stimulated by glucocorticoid or estrogen [6–9]. In the previous study, we have also demonstrated an FGF-like growth promoting activity in the conditioned medium of glucocorticoid-stimulated SC-3 cells as well as androgen-stimulated SC-3 cells [8]. Since many growth factors belonging to the FGF family of proteins have been identified, these biochemical data could not eliminate the possibility that glucocorticoid stimulates the growth of SC-3 cells through the induction of a growth factor other than AIGF. In addition, the growth-stimulatory potency of glucocorticoid on SC-3 cells has been observed to be always lower than that of androgen [6, 8]. These results might suggest that the growth

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stimulation pathway by glucocorticoid is quantitatively or qualitatively different from that by androgen in SC-3 cells. These possibilities could not be examined at the molecular level until recently. The successful cloning of AIGF cDNA and the establishment of an AIGF antisense system [5] enabled us to directly examine these possibilities. Thus, the present study was designed to address the possibility that glucocorticoid-dependent growth of SC-3 cells is mediated through the induction of AIGF.

MATERIALS AND METHODS

Chemicals

[Methyl-^{3}H]thymidine and nonradioactive steroids were obtained as has been described previously [7]. The [α-^{32}P]dCTP (3000 Ci/mmol) was purchased from Amersham (Buckinghamshire, England). BSA (essential fatty acid free) was from Sigma Chemical Co. (St Louis, MO, U.S.A.). The other chemicals used in this study were of analytic grade.

Cells

The SC-3 cell line used in this study was derived from an androgen-dependent mouse mammary SC115 tumor. SC-3 cells were cultured continuously in a maintenance medium composed of Eagle's minimum essential medium (MEM) supplemented with 2% dextran-coated charcoal (DCC)-treated fetal calf serum (FCS) and 10^{-8} M testosterone. Cells were cultured in a humidified incubator in 95% air–5% CO_2 at 37°C.

DNA synthesis

DNA synthesis was measured by [^{3}H]thymidine incorporation in SC-3 cells as has been described previously [7]. It is also outlined briefly in the legend of Fig. 1. The sequences of the AIGF antisense and sense phosphorothioate oligonucleotides were designed to encompass the translation initiation site and were: 5′-GCGGGGGCTGCCCAT3′ (antisense); 5′-ATGGGCAGCCCCCGC3′ (sense) [5].

RNA extraction

SC-3 cells (2×10^6 cells/dish) were plated on 100 mm dishes containing 10 ml of MEM supplemented with 2% DCC-FCS. On the following day, the cells were washed with phosphate-buffered saline (PBS) and the medium was replaced with 10 ml of a serum-free medium [Ham's F-12:MEM (1:1, v/v) containing 0.1% bovine serum albumin] in the absence or presence of various concentrations of testosterone, dexamethasone, estradiol-17β (E_2), or diethylstilbestrol (DES). After incubation for 24 h, the cells were scraped after washing with PBS, and the total cellular RNAs were prepared by the acid guanidinium thiocyanate–phenol–chloroform extraction method [10]. Poly(A)$^+$ RNAs were isolated by Oligo (dT)-latex (Nippon Roche, Tokyo, Japan).

Northern blot analysis

2 μg of poly(A)$^+$ RNAs were electrophoresed in a 1% agarose gel containing 0.66 M formaldehyde and transferred onto a nylon membrane. Hybridization was performed with a ^{32}P-labeled AIGF cDNA probe in 1.5 × SSPE (1 × SSPE = 0.15 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA) containing 1% SDS and 0.5% Blotto at 65°C for 18 h. The blots were subsequently washed with 2 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) containing 0.1% SDS at 50°C for 30 min and with 0.1 × SSC containing 0.5% SDS at 37°C for 30 min. Autoradiography was carried out at −70°C for 48 h. Densitometric analyses of the blots were performed with a MCID (Imaging Research Inc., St Catharines, Canada). The human glyceraldehyde-3 phosphate dehydrogenase (G3PDH) cDNA probe (Clontech, Palo Alto, CA) was used to ensure equal loading of RNA samples in each lane.

RESULTS

Growth-stimulatory effects of androgen, glucocorticoid, or estrogen on SC-3 cells in serum-free medium

The SC-3 cells were cultured in serum-free medium with various concentrations of testosterone, dexamethasone, E_2, or DES for 24 h, and their stimulatory effects on [^{3}H]thymidine uptake into SC-3 cells were examined. Testosterone stimulated the uptake in a concentration-dependent manner and the maximum uptake was observed at 10^{-8} M (Fig. 1). Dexamethasone also stimulated the uptake in a concentration-

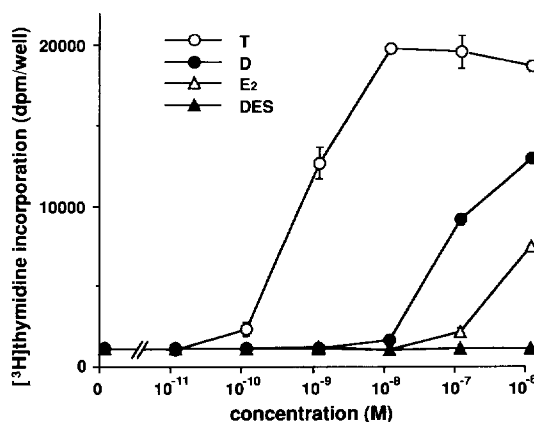

Fig. 1. Stimulatory effects of 10^{-11}–10^{-6} M testosterone, dexamethasone, E_2, or DES on [^{3}H]thymidine incorporation in SC-3 cells in serum-free medium. SC-3 cells were plated onto a 96-well plate (8×10^3 cells/well) containing 0.15 ml of MEM supplemented with 2% DCC-treated FCS. On the following day, the medium was replaced with 0.15 ml of a serum-free medium [Ham's F-12:MEM (1:1, v/v) containing 0.1% BSA] in the absence or presence of various concentrations of testosterone (○), dexamethasone (●), E_2 (△), or DES (▲). After incubation for 24 h, the cells were pulsed with [^{3}H]thymidine (0.15 μCi/0.15 ml per well) for 2 h at 37°C, and the radioactivity incorporated into the cells was measured. The values represent the means ± SE.

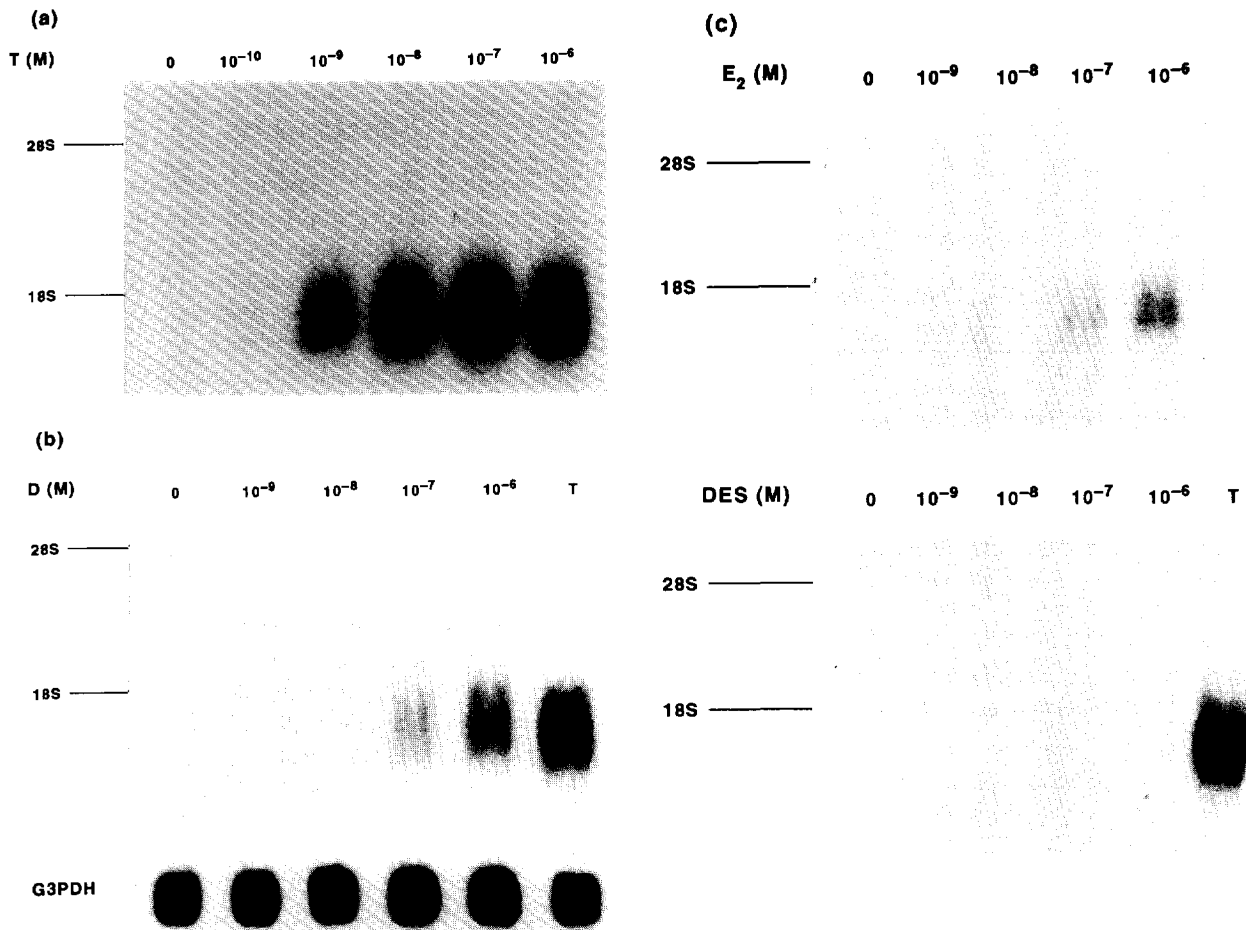

Fig. 2. Induction of AIGF mRNA by testosterone, dexamethasone, E_2 or DES. Northern blot analyses of the expression of AIGF mRNA induced by 10^{-10}–10^{-6} M testosterone, 10^{-9}–10^{-6} M dexamethasone, E_2, or DES are shown. Each lane contained 2 μg of poly(A)$^+$ RNA from SC-3 cells incubated in the absence or presence of various concentrations of testosterone, dexamethasone, E_2, or DES for 24 h. Blots were hybridized with an AIGF cDNA probe derived from pSC17 clone [4]. (a) testosterone; (b) dexamethasone (T: 10^{-8} M testosterone); top, AIGF, bottom, rehybridization with a G3PDH probe; (c) E_2 and DES (T: 10^{-8} M testosterone). The migration positions of 18S and 28S ribosomal RNAs are indicated on the left by lines.

dependent manner. However, the maximum uptake was observed at 10^{-6} M and was about 60% of that induced by testosterone (Fig. 1). E_2 slightly stimulated the uptake at concentrations greater than 10^{-7} M (Fig. 1). In contrast, DES showed no stimulatory effects on SC-3 cells (Fig. 1). These quantitative results were consistent with the previous reports [8, 9].

Induction of AIGF mRNA by androgen, glucocorticoid, or estrogen

We have previously reported that the expression of AIGF mRNA is induced by 10^{-8} M testosterone [4]. In the present study, the expression of AIGF mRNA induced by various concentrations of testosterone, dexamethasone, E_2, or DES was examined. The induction of AIGF mRNA expression by testosterone was observed at concentrations greater than 10^{-10} M, and the maximum effect was observed at 10^{-8} M [Fig. 2(a)]. Dexamethasone at concentrations greater than 10^{-7} M also induced the expression of AIGF mRNA, and the maximum effect was observed at 10^{-6} M [Fig. 2(b)]. Although E_2 at concentrations greater than 10^{-7} M weakly induced the expression of AIGF mRNA, DES showed no effects even at 10^{-6} M [Fig. 2(c)].

Inhibitory effects of AIGF antisense oligonucleotides on the androgen-, glucocorticoid- or estrogen-induced growth of SC-3 cells

In the previous study, we reported that AIGF antisense oligonucleotides could inhibit testosterone-induced DNA synthesis in SC-3 cells [5]. In order to confirm the critical role of AIGF in dexamethasone- or E_2-induced growth of SC-3 cells, we examined the effects of AIGF antisense oligonucleotides on their DNA synthesis. Both dexamethasone- and E_2-induced DNA synthesis were completely inhibited by antisense oligonucleotides at a concentration of 0.5 μM (Fig. 3). Yet, the sense oligonucleotides did not show any inhibitory effects.

Fig. 3. Inhibitory effects of AIGF antisense oligonucleotides on testosterone-, dexamethasone-, or E_2-induced growth of SC-3 cells. SC-3 cells stimulated with 10^{-8} M testosterone, 10^{-6} M dexamethasone, or 10^{-6} M E_2 were incubated without (□) or with 0.5 μM AIGF antisense (■) or sense (▨) oligonucleotides for 24 h. The DNA synthesis in these cells was examined. The values represent the means $\pm$ SE.

Relationship between AIGF mRNA expression and DNA synthesis induced by androgen, glucocorticoid or estrogen

In order to examine whether AIGF mRNA expression levels alone could control the levels of dexamethasone- or E_2-induced DNA synthesis, the relationship between AIGF mRNA expression and DNA synthesis after stimulation with each steroid was investigated. The relative band intensity on Northern blots (Fig. 2) was quantified by densitometric analysis, and then the amount of [^{3}H]thymidine incorporation (Fig. 1) was plotted against the relative band intensity of each concentration of testosterone, dexamethasone, or E_2, taking the value at 10^{-8} M testosterone as 100%. We could clearly see a positive relationship between AIGF mRNA expression and DNA synthesis, irrespective of the hormones used for the stimulation (Fig. 4).

Fig. 4. Relationship between relative AIGF mRNA expression and relative DNA synthesis in SC-3 cells. AIGF mRNA expression and [^{3}H]thymidine incorporation induced by 10^{-10}–10^{-6} M testosterone (○), 10^{-9}–10^{-6} M dexamethasone (●), or E_2 (△) were plotted, taking the values of 10^{-8} M testosterone as 100%. The relative band intensities on the Northern blots were quantified by densitometric analysis.

DISCUSSION

The present study clearly demonstrated that dexamethasone and E_2 could stimulate the expression of AIGF mRNA. The inability of DES to stimulate AIGF mRNA expression indicates that E_2 exerts its action through the androgen receptor. On the other hand, dexamethasone cannot bind to the androgen receptor in SC-3 cells [7]. Thus, the ability of dexamethasone to stimulate AIGF mRNA expression is most likely mediated through the glucocorticoid receptor. Interestingly, AIGF antisense oligonucleotides could block the glucocorticoid-induced enhancement of DNA synthesis. The data obtained by using antisense oligonucleotides needs to be analyzed carefully to exclude their non-specific effects on cell growth. Our previous study has demonstrated that the AIGF antisense oligonucleotides used in this study could specifically inhibit the synthesis of AIGF protein, as these antisense oligonucleotides inhibited the growth of androgen-stimulated cells, but not basic FGF- or FCS-stimulated cells [5]. Moreover, the concentrations (0.5 μM) used in this study were chosen because this concentration could almost completely inhibit the androgen-dependent growth of SC-3 cells without exerting non-specific effects. Thus, the present study demonstrates that glucocorticoid stimulates the growth of SC-3 cells through the induction of AIGF.

A couple of cell lines have been reported to contain multiple receptors for steroid hormones. The growth of each cell line has been shown to be positively or negatively modulated by distinct steroid hormones. The growth of the MCF-7 human breast cancer cell line is stimulated by E_2 [11] or 5α-dihydrotestosterone (DHT) [12]. The B-1 mouse Leydig cell line is also stimulated by E_2 or DHT [13]. The growth of the ZR-75-1 human breast cancer cell line is stimulated by E_2 but inhibited by dexamethasone or DHT [14]. The growth of both the R3327H-G8-A_1 Dunning tumor cell line and the DDT_1MF2 hamster ductus deferens smooth muscle tumor cell line is stimulated by testosterone or E_2 but is inhibited by dexamethasone or triamcinolone acetonide [15, 16]. However, the molecular mechanism of steroid hormone-induced cell growth is unknown in these cell lines other than SC-3 cells. To our knowledge, this is the first report that both glucocorticoid and androgen can exert their growth-stimulatory effect via the induction of a common growth factor (AIGF).

Although both glucocorticoid and androgen can stimulate the expression of AIGF mRNA, the ability of glucocorticoid to stimulate DNA synthesis is lower than that of androgen. To explain these results, a couple of possibilities should be considered. Sato and his co-workers have reported that the bioactivity of

AIGF was modulated by cell-surface or extracellular heparan sulfate [17], heparin [18], or suramine [19]. The stimulation of SC-3 cells with thyroid hormone resulted in their reduced response to AIGF [20]. Thus, one may speculate that glucocorticoid-dependent potentiation of these AIGF modulators results in its attenuated growth-stimulatory ability. However, the present study revealed that the quantitative difference in the growth stimulatory ability between glucocorticoid and androgen could be explained at the AIGF mRNA expression level.

Because the levels of the glucocorticoid receptor and the androgen receptor are similar in SC-3 cells [7], it was somewhat unexpected that the induction level of AIGF mRNA in glucocorticoid-stimulated cells is much lower than that in androgen-stimulated cells. Also, the reason for the relatively high concentration of glucocorticoid required to obtain the maximum induction is unclear. Recently, the promotor regions of androgen responsive genes have been extensively studied, suggesting the presence of a complex mechanism for androgen-dependent gene expression [21, 22]. In view of these reported results, as well as our present data, the cloning and functional analysis of the AIGF promotor is an interesting research project. Our current study is directed toward understanding the expression of this interesting protein.

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