Effect of Androgen on Proliferation of Estrogen-responsive Transformed Mouse Leydig Cells in Serum-free Culture

Yasuko Nishizawa, Bunzo Sato, Kazumasa Nishii, Susumu Kishimoto, and Keishi Matsumoto

Third Department of Internal Medicine [B. S., K. N., S. K.] and Second Department of Pathology [Y. N., K. M.], Osaka University Hospital, Fukushima-ku, Osaka 553, Japan

ABSTRACT

We examined the effects of steroid hormones on the proliferation of transformed mouse Leydig cells (B-1) in serum-free culture condition. Among hormones examined, androgen as well as estrogen enhanced the cell proliferation rate. Hormone binding studies revealed that B-1 cells contained both androgen and estrogen receptors. In addition, androgen-enhanced cell growth was inhibited by antiandrogen, but not by antiestrogen, while estrogen-stimulated cell growth was suppressed by antiestrogen. However, the simultaneous addition of androgen and estrogen did not show an additive effect. Dose-response study on androgen-dependent cell growth revealed that relatively high concentrations (10^{-5} to 10^{-4} M) of dihydrotestosterone were required to obtain the maximum response. This was at least partly explained by the finding that B-1 cells could metabolize dihydrotestosterone into the less active steroids. Finally, B-1 cells were found to grow more rapidly in normal than in castrated male mice.

These results clearly indicate that the proliferation of B-1 cells is stimulated by both androgen and estrogen, which utilize the different receptor systems.

INTRODUCTION

Steroid hormones play an important role in regulating proliferation of transformed as well as nontransformed target cells. Estrogenic hormones have been well known to stimulate the growth of human breast cancer (1). In vitro, androgens inhibit the proliferation of human mammary cancer cells (2). Conversely, androgen-dependent prostate cancer is usually treated with estrogen. These events are considered as an indication that androgen and estrogen are operating in the opposite direction with respect to the tumor growth. However, the increased level of serum androgens has been speculated to be accompanied by a high risk of breast carcinoma (3, 4). Actually, some breast cancers have been proved to contain the AR (5). We have recently reported that the growth of mouse mammary carcinoma (Shionogi carcinoma 115) is enhanced by not only the physiological level of androgen but also the pharmacological level of estrogen (6). To discuss the molecular mechanism of these complicated phenomena, an in vitro cell culture system under well-defined hormonal conditions is desired.

We have previously shown that B-1 cells, the cloned cell line established from estrogen-responsive mouse Leydig cell tumor (T 124958-R), is growth-stimulated by estrogen in the serum-free culture condition (7). In addition, nontransformed Leydig cells in the rodent testes have been documented to have the ER as well as AR (8). Taking these factors into consideration, our aim in the present study was to examine the androgen effect on estrogen-dependent cell line in serum-free culture conditions. Interestingly, androgen stimulated the proliferation of B-1 cells to a level similar to that induced by estrogen. This androgen-dependent proliferation was antagonized by antiandrogens such as cyproterone acetate. To our knowledge, this is the first report showing that there exists a cell line the proliferation of which is stimulated by both estrogen and androgen in serum-free culture.

MATERIALS AND METHODS

Cell Culture. The cell line (B-1) used here was established from estrogen-responsive mouse Leydig cell tumor (T 124958-R) (9) in our laboratory. The cloning methods were reported previously (7). B-1 cells were routinely cultured in MEM supplemented with 10% FCS, 10^{-4} M estradiol (E_{2}) and 0.1 mg/ml bovine insulin.

The protocol used to test the effects of steroids on cell proliferation has been described previously (7) with minor modification. Briefly, cells were plated in four replicate 35-mm culture dishes (10^{5} cells/dish) in MEM supplemented with 1% FCS-DCC. 24 h later (Day 0), the medium was changed to Ham's F-12:MEM (1:1) containing 0.2% (w/v) BSA which was supplemented with various concentrations of steroids. The final concentration of ethanol was always less than 0.01% which was found not to affect the growth of B-1 cells. The solvent was added to control cells. These media were changed every 2 days. After cells were cultured in 95% air-5% CO_{2} for various periods of time in the serum-free culture, the viable cells were counted with the hemocytometer by trypan blue dye exclusion method (7).

ER and AR Assays. B-1 cells were cultured in 10% FCS-DCC-MEM for 4 days to minimize the intracellular residual E_{2} concentration before being harvested with 0.02% (w/v) EDTA-0.01% (w/v) trypsin in PBS. After being washed three times with 5 mM HEPES-MEM, B-1 cells (2-6 x 10^{5}/tube) were incubated with the various concentrations of [^{3}H]-DHT at 37°C for 30 min in the absence or presence of 1 mM unlabeled DHT at a final volume of 0.25 ml to measure total and nonspecific binding as described previously (10). After incubations in 95% air-5% CO_{2} with intermittent shaking, 2 ml of ice-cold 5 mM HEPES-MEM were added to all tubes followed by centrifugation at 1000 rpm for 3 min at 4°C. The pelletted cells were washed three times with 2 ml of 5 mM HEPES-MEM and were added to all tubes followed by centrifugation at 1000 rpm for 3 min at 4°C. The pelletted cells were washed three times with 2 ml of 5 mM HEPES-MEM. To examine the steroid specificity, B-1 cells were incubated with 5 mM [^{3}H]E_{2} or 2 mM [^{3}H]DHT in the presence of the various concentrations of competitors at 37°C for 30 min and treated as described above. To analyze the androgen binding component in the cytosol, B-1 cells were homogenized in TED-Mo-L buffer (5 x 10^{7} cells/ml) using an all-glass homogenizer. The homogenate was centrifuged at 105,000 x g for 1 h at 1°C to obtain the cytosol. The aliquots of the cytosol were incubated with the increasing concentrations of [^{3}H]DHT in the presence or absence of 1 mM unlabeled DHT at 0°C for 2 h, followed by treatment with DCC. The aliquots of the cytosol were also incubated with 2 mM [^{3}H]DHT ± 1 mM unlabeled DHT at 0°C for 2 h for sucrose density gradient analyses. After being treated with DCC, these samples (200 μl) were applied to 5-20% sucrose density gradients prepared in TEM-Mo buffer (low salt) or 0.4 M KCl-TECM buffer (high salt), and centrifuged at 45,000 rpm in a Hitachi RPS-50 rotor at 14-20°C. Fractionation was carried out from 1377.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: AR, androgen receptor; ER, estrogen receptor; MEM, Eagle's minimum essential medium; FCS, fetal calf serum; E_{2}, estradiol-17β; FCS-DCC, fetal calf serum treated with dextran (0.01% w/v) coated charcoal (1% w/v); BSA, bovine serum albumin; TED-Mo-L buffer, 10 mM Tris-1.5 mM EDTA-10 mM Na_{2}MoO_{4}-10 mM diithiothreitol-0.2 mg/ml leupeptin (pH 7.4 at 20°C); TEM-Mo buffer, 10 mM Tris-1.5 mM EDTA-2 mM mercaptoethanol-10 mM Na_{2}MoO_{4}-10 mM diithiothreitol-0.2 mg/ml leupeptin (pH 7.4 at 20°C); TxOH, 4-hydroxytamoxifen; CA, cyproterone acetate; Prog, progesterone; DHT, 5α-dihydrotestosterone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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the bottom of the tube. Fluorescent BSA (4.6 S) was included into all tubes as an internal marker (11).

DHT Metabolism. B-1 cells (2 x 10^6/tube or 2 x 10^4/35-mm dish) were incubated with 10 nM [3H]DHT at the indicated periods of time at 37°C in 95% air-5% CO_2. The reaction was terminated by adding 3 ml of CH_2Cl_2 and vortexing twice for 2 min as described previously (11). The CH_2Cl_2 layer was taken out and evaporated under N_2 gas. After adding the carrier-unlabeled steroids (10 µg), the samples were developed on TLC plates precoated with silica gel 60F254 (Merck, FDR) using CH_3Cl:CH_3OH (99:1; v/v) as a solvent system. Then, the carrier steroids were visualized by spraying 0.1 N H_2SO_4 and heating at 110°C for 30 min. The distribution of the radioactivity on TLC plates was first analyzed by the radiochromatoscanner. The radioactivity which was comigrated with each steroid was then measured by scraping off the silica gel into the counting vials containing the scintillation cocktail as described previously (12).

In Vivo Cell Growth Study. The B-1 cells were permitted to grow to 80% confluency in 10% FCS-0.1 µg/ml insulin-10^{-8} M E_2 in MEM. After being harvested and washed as described above, the cells (5 x 10^5), resuspended in MEM, were injected s.c. into normal or castrated male BALB/c mice. Palpable tumor was measured with calipers (9).

Statistical Analysis. All values were expressed as mean ± SE. Significant differences were estimated by paired Student’s t test.

Materials. [1,2,6,7-3H]E_2 (SA 95 Ci/mmol) and [1,2,4,5,6,7-3H]DHT (SA 110 Ci/mmol) were obtained from New England Nuclear (Cambridge, MA). The radioinert steroids, BSA (crystallized and lyophilized), trypsin, insulin (bovine pancreas), and Norit-A (acid-washed charcoal) were purchased from Sigma Chemical Co. (St. Louis, MO). Dextran T-70 was from Pharmacia Fine Chemicals (Uppsala, Sweden). MEM and Ham’s F-12 were obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). FCS was from Irvine Scientific (Santa Ana, CA). BALB/c mice were purchased from Shizuoka Experimental Animal Farm (Shizuoka, Japan). The other reagents used here were of analytical grade. TxOH was kindly supplied by ICI Pharma (Osaka, Japan).

RESULTS

Effects of Hormonal Steroids on B-1 Cell Proliferation. The ability of various steroids to stimulate the proliferation of B-1 cells was examined in serum-free culture conditions. As depicted in Fig. 1, E_2 (10^{-8} M) growth-stimulated the B-1 cells. Interestingly, DHT (10^{-8} M) also elicited the increased growth rate of B-1 cells. Progesterone and glucocorticoid failed to show a significant effect on B-1 cell proliferation. DHT-induced, but not E_2-induced, enhancement of the cell proliferation was markedly inhibited by antiandrogen (10^{-6} M CA). The cells were then cultured in the presence of the increased concentrations of DHT (Fig. 2). DHT enhanced the proliferation of B-1 cells in a dose-dependent manner. The low concentration (10^{-10} M) of DHT showed the significant increase in the growth rate. However, a relatively high concentration (10^{-7}-10^{-6} M) of DHT was required to obtain the maximum response. Two possibilities were considered to explain these results: (a) this DHT-dependent enhancement of the cell growth is not mediated through putative AR; (b) DHT is metabolized into the hormonally less active steroids during our culture condition. First, we examined the latter possibility. [3H]DHT (10^{-8} M) was added to 35-mm culture dish which contained 5 x 10^5 cells. After incubation for 24 h, total radioactivity was extracted and analyzed as described in “Materials and Methods.” More than 70% DHT added into the dish was found to be metabolized into various steroids. Androstanedione was identified as a major metabolite (Table 1). These observations suggested that the requirement of relatively high concentration of DHT for the maximum response was at least partly due to its metabolism.

AR in B-1 Cells. We then examined the conversion rate of [3H]DHT to the metabolites in the condition which was used for whole cell binding assay. The relatively rapid conversion was also demonstrated in the cell suspension (Fig. 3). This rapid conversion might explain the fact that daily addition of DHT into the culture medium failed to further enhance the cell proliferation rate (data not shown). However, more than 50% radioactivity was identified as unmetabolized DHT after 30 min

<table>
<thead>
<tr>
<th>Concentration of DHT (-log_{10} M)</th>
<th>Cell number/dish (x10^{-4})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Non</td>
</tr>
<tr>
<td>2.0</td>
<td>E_2</td>
</tr>
<tr>
<td>2 x 10^{-10} M</td>
<td>DX</td>
</tr>
<tr>
<td>2 x 10^{-8} M</td>
<td>DHT</td>
</tr>
<tr>
<td>2 x 10^{-6} M</td>
<td>Prog</td>
</tr>
<tr>
<td>2 x 10^{-4} M</td>
<td>E_2+CA</td>
</tr>
<tr>
<td>2 x 10^{-2} M</td>
<td>DHT+CA</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of various steroids on B-1 cell growth. B-1 cells (10^5 cells/35-mm dish) were plated and cultured for 7 days in the serum-free medium supplemented with 10^{-8} M steroids ± 10^{-6} M CA. The viable cells were counted.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>No cell</th>
<th>% Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHT (unmetabolized)</td>
<td>26.9%</td>
<td>90%</td>
</tr>
<tr>
<td>Androstanedione</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>Testosterone</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>Androstanedione</td>
<td>62.3%</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>5α-Androstane-3α,17β-diol</td>
<td>7.2%</td>
<td>2.4%</td>
</tr>
</tbody>
</table>

Table 1 DHT metabolism in the condition used for cell growth experiments

[3H]DHT (10 nm) was added to dishes with or without B-1 cells (2 x 10^5 cells/35-mm dish). After incubation for 24 h, the metabolites of DHT were analyzed as described in “Materials and Methods.”
EFFECTS OF ANDROGEN AND ESTROGEN ON CELL GROWTH

Incubation Time (min)

Fig. 3. Time course study on DHT metabolism in the condition used for whole cell binding assay. B-1 cell suspension (2 x 10^6 cells/0.25 ml MEM) was incubated with 10 nM [3H]DHT at 37°C for the indicated periods of time. These samples were subjected to analyses of DHT metabolites as described in "Materials and Methods." Incubation. Therefore, B-1 cells were incubated with [3H]DHT for 30 min for whole cell binding assay. The binding data obtained by incubation with 0.15–10 nM [3H]DHT in the absence or presence of 1 μM unlabeled DHT were analyzed according to Scatchard method (13) (Fig. 4, left). The apparent dissociation constant (Kd) and maximum binding sites were calculated to be 1.55 ± 0.25 nM and 12.6 ± 1.5 fmol/10^6 cells (N = 5), respectively. Although these values were compatible with the idea that B-1 cells contained AR, accurate measurement of AR seemed to be difficult due to the ligand metabolism. To obviate this metabolism problem, the binding data were also obtained by incubating the cytosolic cell extract with [3H]DHT at 0°C for 2 h (Fig. 4, right). The apparent Kd and maximum binding sites were also observed to be 2 nM and 25 fmol/mg protein, respectively (n = 2). To confirm the presence of AR in B-1 cells, the steroid specificity of the binding components was then investigated. Since B-1 cells were reported to have ER from our laboratory (9), the experiments using [3H]E2 as a radioactive ligand were also conducted. ER binding was found to be specific for estrogens as well as Totorone (Fig. 5, left). On the other hand, the addition of unlabeled DHT or testosterone demonstrated a concentration-dependent decrease in [3H]DHT binding to B-1 cells. E2, CA, androstanedione and Prog also competed but in a lesser degree than DHT or testosterone, while DES, Totorone, and Dex did not compete for [3H]DHT binding even at μM concentrations (Fig. 5, right). Our preliminary study on the subcellular localization of [3H]DHT binder revealed that more than 80% binding activity was recovered into the cytosol fraction upon cell homogenization (data not shown). Therefore, sucrose density gradient analyses of the cytosolic [3H]DHT binder were performed (Fig. 6). To minimize the proteolytic activity probably present in B-1 cells, cell homogenization was carried out in the buffer containing 10 mM Na2MoO4 and 0.2 mg/ml leupeptin (14). The sedimentation constants in the low and high (0.4 M KCl) conditions were determined to be 12S and 6S, respectively. Although these

Fig. 4. Scatchard analysis of the data on the specific binding of [3H]DHT to B-1 cells or their cytosol. Intact B-1 cells (left) or their cytosol (right) were incubated with various concentrations of [3H]DHT ± 1 μM unlabeled DHT as described in "Materials and Methods." The specific binding was analyzed according to the Scatchard Method.

Fig. 5. Steroid specificity of E2 and DHT binding to B-1 cells. The binding specificity of B-1 cells for E2 (left) or DHT (right) was studied as described in "Materials and Methods." These values are mean of two separate experimental results.

Fig. 6. Sucrose density gradient analyses of [3H]DHT binding component in B-1 cell cytosol. The cytosol was incubated with 2 nM [3H]DHT in the absence (B) or presence (O) of 1 μM unlabeled DHT at 0°C for 2 h. The sedimentation profiles were analyzed in the low salt (A) or high salt (B) condition.
sedimentation constant values were somehow larger than those previously reported in androgen sensitive cells (15, 16), the sedimentation profiles of steroid receptors have been well known to be influenced by many factors (17, 18). Collectively, all data on [3H]DHT binding clearly indicated the presence of AR in B-1 cells.

AR-mediated Growth. As already described in Fig. 1, DHT-dependent growth of B-1 cells was inhibited by CA. To confirm further that this androgen effect is mediated through AR, the dose-response study was performed (Fig. 7A). The enhancement of B-1 cell growth elicited by 10^{-8} M DHT was significantly suppressed by 10^{-8} M CA. The increase in DHT concentrations reversed the antiandrogenic ability of CA, excluding the possibility that CA acted as a nonspecific toxic substance. The growth enhancement induced by 10^{-9} M DHT was not influenced by TxOH at the concentrations of less than 10^{-8} M. The growth inhibition by high concentrations of TxOH might be mediated through steroid receptor-independent process (7). This inhibitory effects of high concentrations of TxOH could not be antagonized by high concentrations (10^{-6} M) of DHT (data not shown). On the other hand, estrogen-induced enhancement was blocked by 10^{-8} M TxOH. This inhibition induced by 10^{-8} M TxOH was reversed by 10^{-7} M E2, while the inhibitory effect of 10^{-7} M TxOH could not be antagonized by 10^{-7} M E2, again suggesting that cell growth inhibition induced by 10^{-7} M TxOH is not mediated through steroid receptor. These results indicating the presence of two different pathways for steroid hormones in B-1 cells led us to study the additive effects of estrogen and androgen on cell growth. However, E2 (10^{-9} M) failed to further enhance the growth of B-1 cells stimulated by 10^{-6} M DHT. Moreover, the experimental results on the daily rate of proliferation during the 10-day culture period showed the lack of the additivity of E2 and DHT (Fig. 7D).

The possibility was also addressed that B-1 cells showed androgen-dependent growth in vivo, although whole animal treatment regimens are well known to change numerous hormonal environments. The B-1 cells were inoculated s.c. into intact and castrated male mice (Fig. 8). In this particular experiment, the incidence of tumor growth in these groups was not so high (five of 10 for intact mice, seven of 10 for castrated mice). When the growth of the palpable tumors was measured, however, castration significantly slowed the tumor growth, suggesting that the physiological levels of androgen are able to enhance the proliferation of B-1 cells in vivo despite their ability to metabolize androgen into less active steroids.

DISCUSSION

The present study clearly shows that B-1 cells which have been selected as an estrogen-sensitive cell line are potentially growth-stimulated by androgen. The actions of androgen in the estrogen-responsive cells were complicated. Physiological doses of androgen inhibit the estrogen-induced events in rat uteri (19) and dimethylbenz(a)anthracene-induced rat mammary tumor (20). Maclndoe and Etre (21) also reported that the E2-induced augmentation of cytosolic progesterone receptor levels in MCF-7 cells could be inhibited by physiological concentrations of androgens. This antiestrogenic action by androgens was blocked by the addition of antiandrogens such as CA, suggesting that this effect was mediated by AR. Using a human hepatoma cell line, Tam et al. (22) proposed that the ability of testosterone to suppress the estrogen-induced synthesis of apolipoprotein might be elicited by AR-dependent induction of a cytoplasmic moderate-affinity estrogen-binding component. On the other
hand, pharmacological doses of androgen have been documented to act as an estrogen agonist through ER in the estrogen target tissues (23, 24). In our serum-free culture system, androgen stimulated the proliferation of estrogen-sensitive B-1 cells through the AR-dependent process, judged by the present findings that B-1 cells are AR-positive and their androgen-dependent growth is inhibited by antiandrogen. We have previously shown that the growth of mouse mammary tumor (Shionogi Carcinoma 115) is markedly stimulated in vivo by androgen as well as estrogen (6). The cloned cell line (SC-3) established from Shionogi Carcinoma 115, however, failed to show the growth-stimulatory effects of estrogens even at the very high concentrations in the serum-free culture conditions, although SC-3 cells contain both AR and ER (25). 

E2 as well as Prog was reported to growth-stimulate an androgen-dependent cell line derived from the Dunning R3327H adenocarcinoma (26). An intriguing finding was that these cells had no demonstrable ER. In addition, many in vitro studies on the effects of sex hormones have been carried out in the presence of FCS. Even after being treated with DCC, FCS has been found to contain the conjugated form of sex hormones, sex hormone binding globulin, and many other unknown factors which might influence experimental results (27, 28). To our knowledge, this is the first report showing that both estrogen and androgen stimulate the proliferation of cells containing ER as well as AR.

AR present in B-1 cells is worth discussing. The relatively rapid conversion of DHT mainly to androstanedione hindered the accurate measurement of AR. Even if [3H]methyltrienolone was used as a ligand, some metabolism was found to occur (data not shown). We have also shown that mouse Leydig cell tumors are capable of metabolizing Prog into inactive steroids without forming the active androgens (29). This fact may be attributable to the lack of the synandrogenic effects of Prog (30) on B-1 cell proliferation. Nevertheless, the data on [3H]-DHT binding characteristics and sucrose density gradient analyses clearly demonstrated the presence of AR in B-1 cells. The sedimentation constants of AR in B-1 cells were found to be larger than those in various androgen target cells as described previously. Since our previous report has documented that ER in the cultured transformed Leydig cells shows some unique characters (10, 31), the possibility might exist that AR somehow deviates from that present in nontransformed target tissues. In addition, it should be mentioned that AR is extremely sensitive to proteolytic fragmentation (15).

An attempt to elicit the synergistic effects of E2 and DHT was unsuccessful. This was not attributable to the possibility that B-1 cells already receive the maximum stimulation by one hormone, since the addition of growth promoting factors such as FCS to E2-supplemented medium further accelerates the growth of B-1 cells. In this relation, Moore et al. (32) have demonstrated that estrogen increases AR contents in the dog prostate leading the target cells to enhanced androgen sensitivity. In contrast, estrogen-induced down-regulation of AR has been reported to occur in MCF-7 cells (33). Another possibility to explain the lack of synergism between E2 and DHT is that these sex hormones share the common pathway beyond their receptor levels to stimulate cell proliferation. In this regard, glucocorticoid-receptor complexes have been reported from our laboratory to induce androgen-dependent proteins in SC-3 cells (34). These possibilities are currently under investigation.

Finally, we should discuss the present data with relevance to endocrine therapies for human cancer. Estrogen has been widely used to treat prostate cancer, while androgen has sometimes been administered to women with breast cancer. Identification of transformed cells showing both estrogen- and androgen-responsiveness raises the possibility that some endocrine therapies result in unfavorable responses. Thus, the growth-stimulatory mechanism of androgen and estrogen in a cloned cell line should be clarified. B-1 cells would provide us with the suitable model to investigate the cancer growth enhanced by sex hormones.

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