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CONTRIBUTION OF AROMATASE TO THE DEOXYRIBONUCLEIC ACID SYNTHESIS OF MCF-7 HUMAN BREAST CANCER CELLS AND ITS SUPPRESSION BY AROMATASE INHIBITORS*

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Summary—We have studied the effects of various steroids on DNA synthesis in MCF-7 human breast carcinoma cells, which have aromatase activity and which exert an oestrogen receptor-mediated growth, to assess the significance of intracellular aromatase on growth stimulation as well as inhibition by aromatase inhibitors. The cells were cultured for 96 h in phenol red-free medium containing 10% charcoal-treated fetal bovine serum and test reagents and pulse-labelled with [^{3}H]thymidine. Physiological concentrations of oestradiol, oestrone, testosterone (T) and androstenedione (AD) stimulated thymidine incorporation. However, oestrone-sulphate and dihydrotestosterone (DHT) only stimulated at concentrations greater than the physiological levels. T and DHT stimulation was blocked by tamoxifen, but not by cyproterone acetate, suggesting that the stimulation was mediated via the oestrogen receptor but not by the androgen receptor. Stimulation by T and AD was reduced by aminoglutethimide and 14α-hydroxy-4-androstene-3,6,17-trione, both of which inhibit aromatase activity, however, stimulation by nonaromatizable DHT was not reduced by the inhibitors, suggesting that androgens were converted by the intracellular aromatase to oestrogens which stimulated the thymidine incorporation. It is suggested that intracellular aromatase significantly contributes to the stimulation of DNA synthesis and that aromatase inhibitors suppress the stimulation.

INTRODUCTION

In postmenopausal women, oestrogens produced from adrenal androgens through an aromatase enzyme system which is located predominantly in extraglandular tissues, such as fat, muscle and other tissues, play an important role in stimulation of the growth of hormone-dependent breast cancers [1]. This is supported by the clinical usefulness of antioestrogens [2] and aromatase inhibitors [3, 4] for the treatment of breast cancer patients. Although circulating oestrone (E_1) and oestradiol (E_2) concentrations in postmenopausal women are insufficient for saturation of the breast cancer oestrogen receptor [5], intracellular E_1 and E_2 concentrations in breast cancer tissue are more than 10 times higher than those in plasma [6–8]. A number of studies have demonstrated that 30 to 60% of breast carcinomas have substantial aromatase activity [9–14] higher than that of normal breast tissue and surrounding adipose tissue [15]. This indicates that the carcinoma cells take circulating androgens and convert them to oestrogens through intracellular aromatase. Some clinical studies have suggested that breast cancers with high aromatase activity have a better response to treatment with aromatase inhibitors than tumours in which aromatase is undetectable [9, 16], Another study has shown that breast cancers with detectable aromatase have high histological grade, and that such patients have a long survival period after relapse [13]. On the other hand, Santner *et al.* [17, 18] suggested that aromatization provides only a minor portion of intracellular oestrogen, and that the hydrolysis of circulating oestrone-sulphate (E_1-S), which is 10 times higher than E_1 [19], by intracellular sulphatase is the main source of *in situ* oestrogen production.

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Abbreviations: E_1, oestrone; E_2, oestradiol; E_1-S, oestrone 3-sulphate; AD, androstenedione; TAM, tamoxifen; CPA, cyproterone acetate; AG, aminoglutethimide; 14α-OHAT, 14α-hydroxy-4-androstene-3,6,17-trione; T, testosterone; DHT, dihydrotestosterone.

This investigation was undertaken to clarify the extent to which oestrogens produced by intracellular aromatase contribute to the stimulation of tumour growth, using MCF-7 human breast carcinoma cells as a model. MCF-7 cells exhibit oestrogen-dependent growth via the oestrogen receptor when they are exposed to oestrogens [20, 21]. MCF-7 cells also have the ability to hydrolyse oestrogen sulphates to unconjugated oestrogens [22, 23] and to aromatize androgens to oestrogens [24]. The latter implies a pathway through which exogenous androgens are converted by intracellular aromatase to oestrogens, which then interact with the oestrogen receptor. Androgens have been shown to stimulate the growth of MCF-7 cells [25], however, it has been suggested that this phenomenon is caused mainly by pharmacological concentrations of androgens interacting directly with the oestrogen receptor [26]. In the present study we compared the biological activitities of androgens and conjugated and unconjugated oestrogens on the stimulation of DNA synthesis in MCF-7 cells using [^{3}H]thymidine incorporation as an endpoint. We validated and used a sensitive assay for aromatase that measures the amount of [^{3}H]water formed during the conversion of [1β-^{3}H]androstenedione (AD) to E_1.

MATERIALS AND METHODS

Chemicals

[1,2,6,7-^{3}H]AD, [1β-^{3}H]AD, and [^{3}H]thymidine were purchased from Dupont-New England Nuclear (Boston, MA). [4-^{14}C]E_1 and [4-^{14}C]E_2 were purchased from Amersham (Bucks., England). Nonradioactive steroids and tamoxifen (TAM) were purchased from Sigma (St Louis, MO). Cyproterone acetate (CPA) was provided by Schering (Germany). Aminoglutethemide (AG) was provided by Ciba-Geigy (Summit, NJ). 14α-Hydroxy-4-androstene-3,6,17-trione (14α-OHAT) [27] was a gift from Dr Yoshihama of Snow Brand Milk Products (Tochigi, Japan).

Cell culture and [^{3}H]thymidine incorporation

The MCF-7 human breast carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD). HHUA human endometrial carcinoma cell line [28] was a gift from Dr Ishiwata of Ishiwata Obstetrics and Gynecologic Hospital (Mito, Japan). MCF-7 and HHUA cells were grown in plastic culture flasks in Eagle's Minimal Essential Medium with Earle's salts and Ham's F-12 (Gibco, Grand Island, NY), respectively. The growth media were supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone. Cells were cultured in a humidified atmosphere of 5% CO_2–95% air. Cells at preconfluence were subcultured by treatment with phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} containing 0.125% trypsin and 0.01% EDTA and inoculated into 96-well culture plates at a density of 2×10^4 cells/0.2 ml/well. The experimental medium was the same but without phenol red due to potential oestrogen receptor interaction [29]. Fetal bovine serum was treated twice with charcoal (6.25 mg/ml) and dextran T-70 (0.625 mg/ml) by incubation at 56°C for 30 min to remove endogenous steroids.

After a 24 h preincubation, the cells were incubated in medium containing the compound to be tested. The medium was renewed every 24 h. Test reagents were dissolved in ethanol and added to the medium, with a final concentration of ethanol of 0.1%. On the final day, wells were washed with fetal bovine serum-free medium, medium containing [^{3}H]thymidine (0.67 μCi/well) was added, and cells were pulse-labelled for 1 h. The medium was removed and the wells washed with ice-cold 67 mM PBS (pH 7.4). The cells were fixed with ethanol, washed twice with 5% trichloroacetic acid, dissolved in 1 N NaOH, and an aliquot was taken for counting on a Tri Carb Packard liquid scintillation spectrophotometer Model 3375. The blank (without cells) was 30 dpm, or $< 1\%$ of the control.

Aromatase assay

Aromatase activity was measured by the tritiated water method [30, 31]. Preconfluent MCF-7 cells in 60-mm Petri dishes were incubated with 1 ml of medium containing 100 U/ml penicillin, 100 μ/ml streptomycin, 0.25 μg/ml fungizone and [1β-^{3}H]AD (6.0×10^6 dpm, 100 pmol) at 37°C in a humidified atmosphere of 5% CO_2–95% air for 1–4 h. The medium was transferred to a test tube, 0.2 ml of 20% trichloroacetic acid and 1.0 ml of 5% charcoal were added, and the mixture was incubated at 37°C in air for 30 min. The mixture was centrifuged and the supernatant was filtered through a cotton-plugged dispo-pipette. The amount of [^{3}H]water in the eluate was assessed based on the theory that 1β-^{3}H is eliminated

during aromatization of the [1β-^{3}H]AD substrate (75% release into water) [30].

For validation of the tritiated water method, aromatase activity was determined by the product isolation method. Preconfluent cells in two 150-cm^2 flasks were incubated with 16 ml of medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone and [1,2,6,7-^{3}H]AD (3.5×10^8 dpm, 1.6 nmol) at 37°C in a humidified atmosphere of 5% CO_2–95% air for 4 h. The analytical procedures for chromatographic separation were identical to those described in our earlier publication [32]. The medium was mixed with 50 ml of ethyl acetate containing [4-^{14}C]E_1 and [4-^{14}C]E_2 (10,000 dpm, 250 μg each). The mixture was extracted with ethyl acetate (50 ml × 3) and the extract was washed with water (10 ml × 3). The organic phase was evaporated and submitted to Bio-Rad AGI-X2 (50–100 mesh) column chromatography. The phenolic fractions thus obtained were purified by TLC (cyclohexane–ethyl acetate, 2:1, v/v; and chloroform–ethyl ether, 4:1, v/v). [^{3}H, ^{14}C]E_1 and [^{3}H, ^{14}C]E_2 were cocrystallized to constant specific activity and ^{3}H/^{14}C ratio of the crystals. The final crystals were acetylated with acetic anhydride in pyridine and cocrystallized.

Receptor assay

Preconfluent cells were washed three times with 10 mM Tris–HCl buffer (pH 7.4) containing 2 mM mercaptoethanol and 10 mM Na_2MoO_4, removed with a scraper, collected in a centrifuge tube, and washed again with the buffer. The cells were homogenized in the buffer and centrifuged at 50,000 *g* for 60 min. The cytosol concentrations of oestrogen and progesterone receptors were determined by the corresponding specific sandwich enzyme immunoassay kits purchased from Abbott (Abbott Park, IL).

Others

Protein concentrations were determined by a modification of Lowry's method [33] using bovine serum albumin as the standard.

Data are expressed as the mean ± SEM. Statistical analyses were performed by unpaired Student's *t* tests.

RESULTS

Validation of the tritiated water assay

MCF-7 cells in 60-mm Petri dishes were incubated with [1β-^{3}H]AD for various periods of time. As shown in Fig. 1, the [^{3}H]water release was linear with time up to 3 h. When the incubation was performed in the presence of 5 μM progesterone to block 5α-reductase activity, the rate of [^{3}H]water release was also linear for up to 3 h and was slightly greater than that in the absence of progesterone. The [^{3}H]water production of the blank (no cell) incubation and HHUA human endometrial carcinoma cells which had no detectable aromatase activity were negligible and equaled the theoretical zero time conversion. We therefore set the incubation time at 3 h and the radioactivity of the blank incubation was subtracted from each sample. With this method the aromatase activity

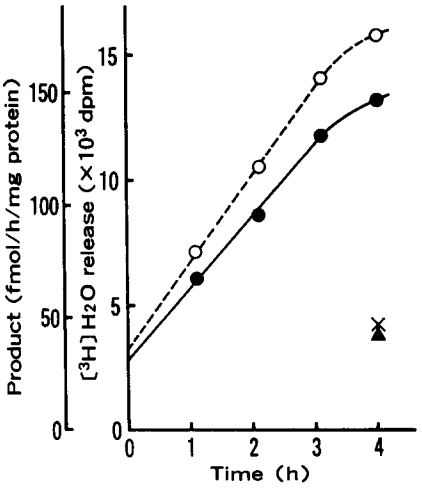

Fig. 1. Time-course of [^{3}H]water release from [1β-^{3}H]AD by MCF-7 cells. Cells preconfluent in 60-mm Petri dishes were incubated with medium containing [1β-^{3}H]AD (6.0×10^6 dpm, 100 nM) in the presence (○) or absence (●) of 5 μM progesterone. ▲, [^{3}H]water release from [1β-^{3}H]AD by HHUA human endometrial carcinoma cells; ×, no cell blank. Data are the mean of six determinations in three independent experiments.

Table 1. Radiochemical purity of [^{3}H]E_1 and [^{3}H]E_2 formed from [1,2,6,7-^{3}H]AD by MCF-7 cells[a]

^{3}H/^{14}C ratio			
Cocrystallization			
E_1		E_2	
1	51	1	7.1
2	51	2	7.2
3	48	3	7.5
E_1 acetate[b]		E_2 diacetate[b]	
1	53	1	7.1
2	51	2	7.1

[a]MCF-7 cells preconfluent in two 150-cm^2 flasks were incubated at 37°C for 3 h with medium containing [1,2,6,7-^{3}H]AD (3.5×10^8 dpm, 100 nM). The medium was extracted with ethyl acetate containing [^{14}C]E_1 and [^{14}C]E_2 (10,000 dpm, 250 μg each), and chromatographed on a Bio-Rad AGI-X2 column and TLC. The E_1 and E_2 fractions were each mixed with 50 mg carrier steroid and cocrystallized.

[b]The third crystals of E_1 and E_2 were acetylated and cocrystallized.

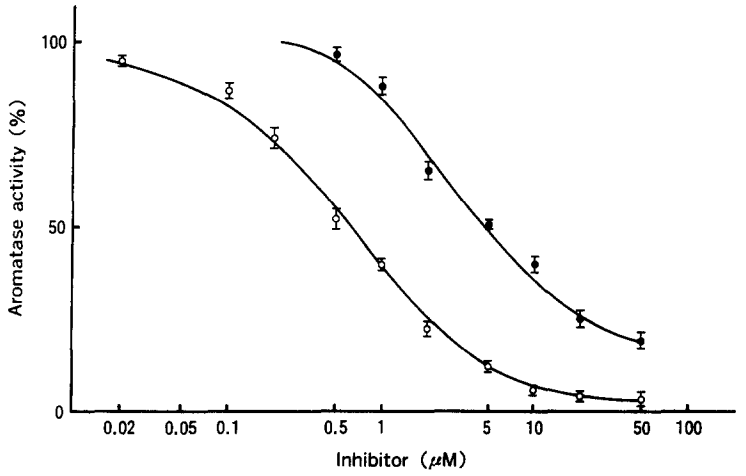

Fig. 2. Concentration-dependent inhibition of aromatase activity of MCF-7 cells by aromatase inhibitors. MCF-7 cells were incubated for 3 h with medium containing [1β-^{3}H]AD (6.0×10^6 dpm, 100 nM), and increasing concentrations of AG (●) or 14α-OHAT (○). Data are expressed as the mean ± SEM ($n = 3$).

of untreated MCF-7 cells was calculated to be 32 fmol/h/mg protein.

To validate the tritiated water method for use with MCF-7 cells, aromatase activity was determined by the product isolation method. Cells were incubated with the equivalent concentration (100 nM) of [1,2,6,7-^{3}H]AD. The final identity of E_1 and E_2 was confirmed by acetylation and cocrystallization to a constant $^3H/^{14}C$ ratio (Table 1). Based on the final $^3H/^{14}C$ ratios the amounts of E_1 and E_2 produced were calculated to be 0.17 and 0.02% of the original substrate, accounting for 23 and 3 fmol/h/mg protein, respectively. These values were comparable to the aromatase activity determined by the tritiated water method, indicating that the majority of [^{3}H]water produced from [1β-^{3}H]AD by MCF-7 cells was derived during the aromatization of the substrate.

As shown in Fig. 2, aromatase activity determined by the tritiated water method was inhibited by AG and 14α-OHAT in a concentration-dependent manner with IC_{50} values of 5.0×10^{-6} M and 5.0×10^{-7} M, respectively.

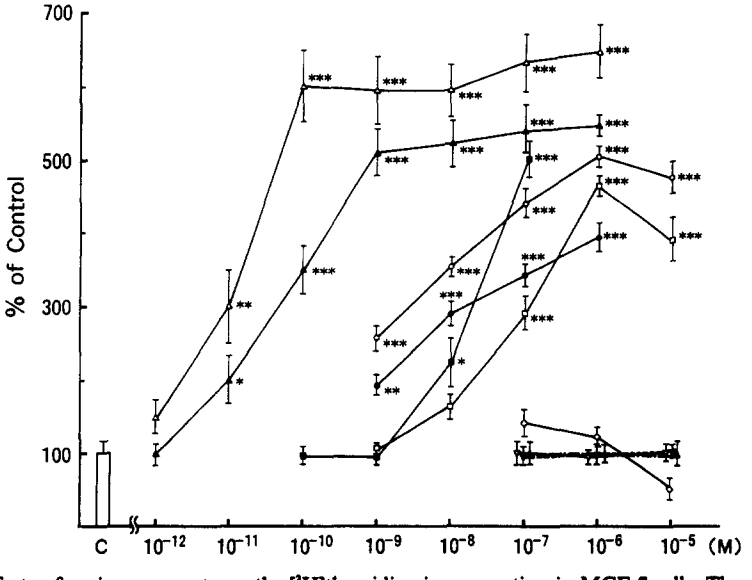

Fig. 3. Effects of various reagents on the [^{3}H]thymidine incorporation in MCF-7 cells. The cells (2×10^4 cells/ 96-well) were cultured first in phenol red-free medium containing 10% charcoal-treated fetal bovine serum for 24 h, and subsequently for 96 h in the same medium containing the indicated concentration of each reagent, with daily renewal of the medium. The cells were then pulse-labelled with [^{3}H]thymidine (0.67 μCi/well) for 1 h. △, E_2; ▲, E_1; ○, T; ●, AD; □, DHT; ■, E_1-S; ◇, TAM; ▲, CPA; ▼, AG; and ▽, 14α-OHAT. Data are the mean ± SEM of 16 determinations in four independent experiments. *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$ vs control.

The cytosol oestrogen and progesterone receptor levels in untreated MCF-7 cells were 60 and 13 fmol/mg protein, respectively.

[^{3}H]Thymidine incorporation

MCF-7 cells were incubated for 24–120 h in the medium containing various test compounds. The amount of ^{3}H]thymidine incorporation increased linearly with time under our assay conditions (data not shown), so the incubation time was set at 96 h. Figure 3 shows the [^{3}H]thymidine incorporation after the addition of individual test compound. As expected, E_2 stimulated thymidine incorporation in a dose-dependent manner at concentrations up to 10^{-10} M, at which the stimulation reached 600% of the control and remained at that level at concentrations between 10^{-10} and 10^{-6} M. E_1 also stimulated incorporation of thymidine, reaching a maximum of 500% at 10^{-9} M, but the stimulation was significantly lower than that of E_2 at every concentration tested. In contrast, E_1-S had no effect on stimulation at concentrations up to 10^{-9} M, the physiological circulating level in postmenopausal women, but stimulated at 10^{-8} and 10^{-7} M. T, AD and

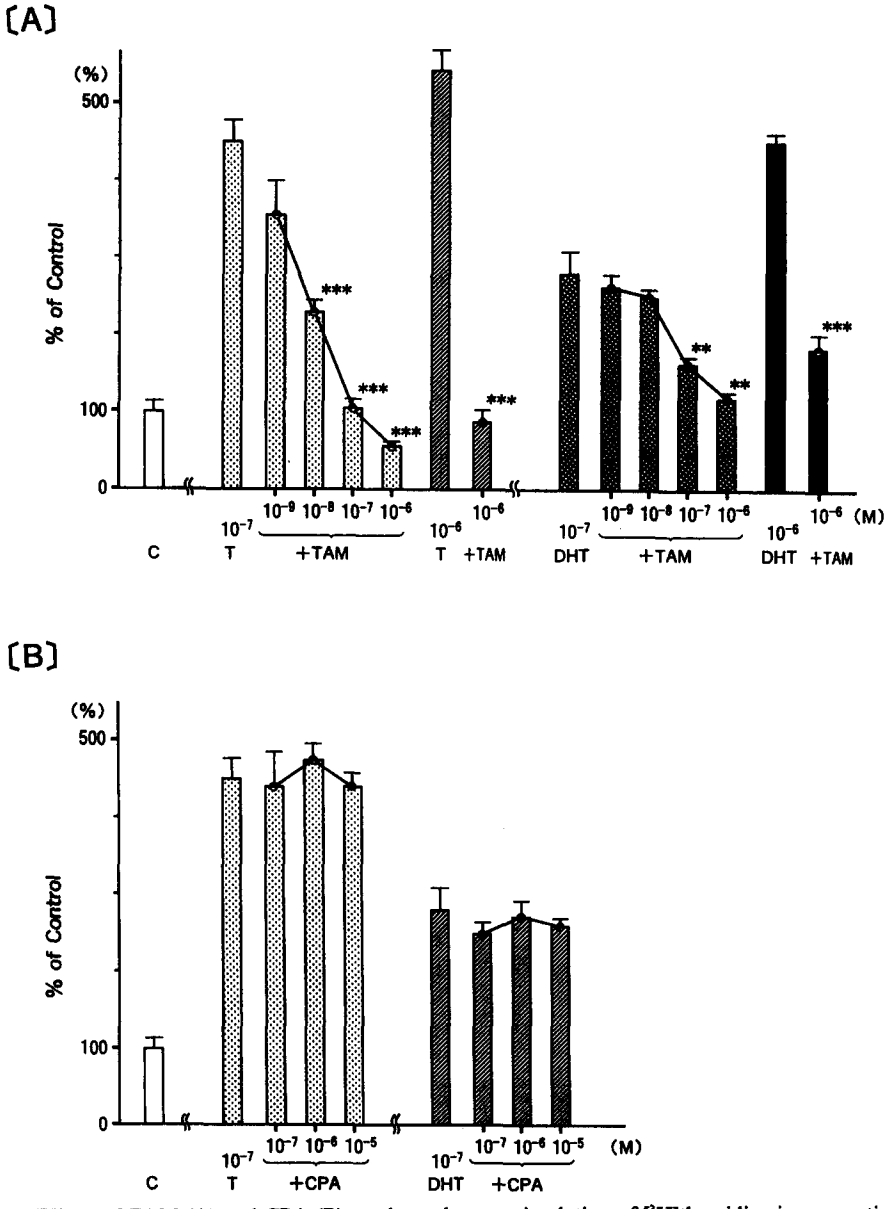

Fig. 4. Effects of TAM (A) and CPA (B) on the androgen stimulation of [^{3}H]thymidine incorporation in MCF-7 cells. The experimental conditions were identical to those for Fig. 3. Data are the mean ± SEM of 16 determinations in four independent experiments. $^{**}P < 0.01$; and $^{***}P < 0.001$ vs androgen alone.

dihydrotestosterone (DHT) also stimulated thymidine incorporation in a dose-dependent manner in the concentration range between 10^{-9} and 10^{-6} M. Stimulation by T was 260% at 10^{-9} M, the physiological circulating level. This was significantly higher than that by AD or DHT. Single administration of TAM, the androgen receptor inhibitor CPA, or aromatase inhibitor AG or 14α-OHAT, did not affect thymidine incorporation at the concentration between 10^{-7} and 10^{-5} M. That CPA had no effect on stimulation was in contrast to the result of Hackenberg *et al.* [34] who reported that it was stimulatory at 10^{-7} M.

To determine the androgen stimulatory mechanism, TAM or CPA was administered in combination with androgens. Stimulation by T and DHT was blocked in a concentration-dependent manner by the simultaneous administration of TAM, and was almost completely offset by 10^{-6} M TAM [Fig. 4(A)]. On the other hand, stimulation by T and DHT was not reduced by simultaneous addition of 10^{-7} to 10^{-5} M CPA [Fig. 4(B)]. These results suggest

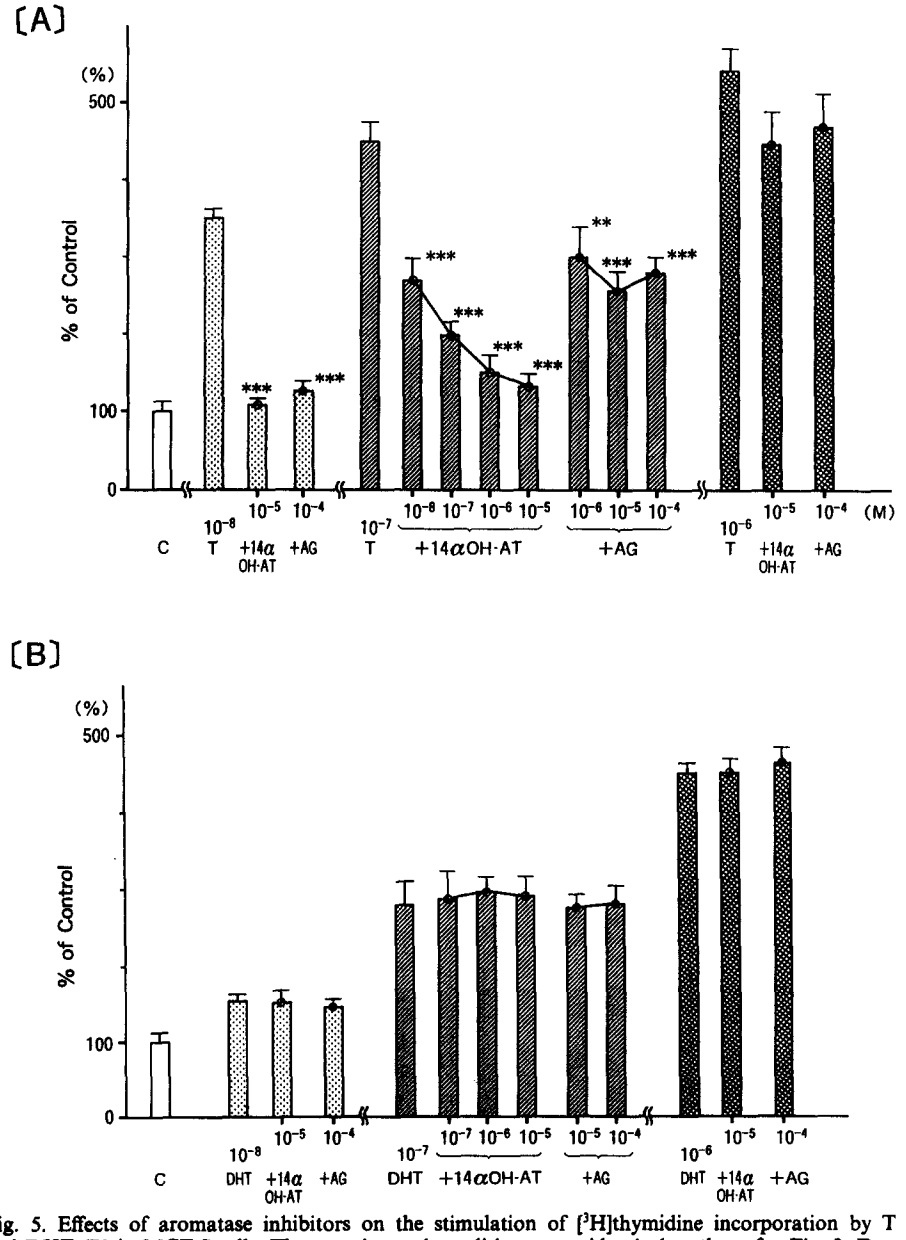

Fig. 5. Effects of aromatase inhibitors on the stimulation of [^{3}H]thymidine incorporation by T (A) and DHT (B) in MCF-7 cells. The experimental conditions were identical to those for Fig. 3. Data are the mean ± SEM of 16 determinations in four independent experiments. $^{**}P < 0.01$; and $^{***}P < 0.001$ vs T alone.

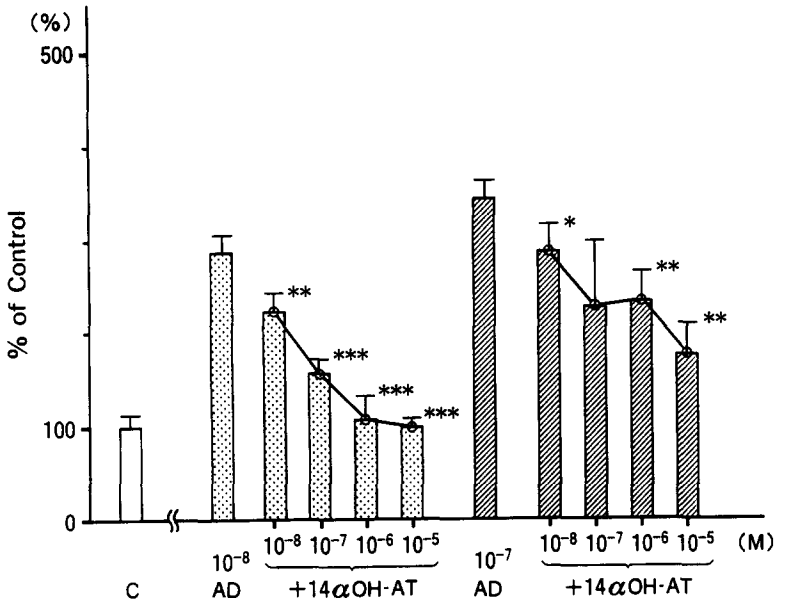

Fig. 6. Inhibition of the AD stimulation of [^{3}H]thymidine incorporation by 14α-OHAT in MCF-7 cells. The experimental conditions were identical to those for Fig. 3. Data are the mean ± SEM of 16 determinations in four independent experiments. *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$ vs AD alone.

that the stimulation by androgens was mediated via the oestrogen receptor but not via the androgen receptor, supporting the previous report that T and DHT at greater than physiological concentrations stimulate the growth of MCF-7 through crossreaction of the androgens with the oestrogen receptor [26].

We then examined the mechanism by which E_2 converted from T by intracellular aromatase binds to the oestrogen receptor. As shown in Fig. 5(A), the stimulation of thymidine incorporation by T was reduced by simultaneous administration of the aromatase inhibitor, 14α-OHAT or AG. This reduction was concentration-dependent on the mechanism-based inhibitor 14α-OHAT. Stimulation by 10^{-8} M T was completely blocked to the control level by aromatase inhibitors. Stimulation by 10^{-7} M T was also blocked completely by 14α-OHAT and decreased to 70% by AG. At 10^{-6} M T however, little reduction was observed following simultaneous administration of the aromatase inhibitors. In contrast, the aromatase inhibitors were completely ineffective in affecting reduction of the stimulation by the nonaromatizable DHT [Fig. 5(B)]. At lower concentrations (10^{-8} and 10^{-7} M), crossreaction of T and DHT with the oestrogen receptor was minimal. Therefore it appears that the portion blocked by aromatase inhibitors in the T stimulation of thymidine incorporation or the difference in incorporation between T and DHT can be attributed to the stimulation of DNA synthesis by E_2 which had been derived from T through intracellular aromatase. The simultaneous administration of 14α-OHAT also blocked the stimulation of thymidine incorporation by AD in a concentration-dependent manner (Fig. 6), indicating that AD was converted to E_1 and E_2 by intracellular aromatase and that the oestrogens interacted with the oestrogen receptor.

DISCUSSION

The present study demonstrated that in MCF-7 cells, physiological concentrations of T are converted by the aromatase enzyme to oestrogens. These oestrogens bind to the oestrogen receptors, contributing significantly to the stimulation of DNA synthesis.

Previous studies have shown that androgens at concentrations greater than physiological levels stimulate the growth of MCF-7 cells by crossreaction with oestrogen receptors but not with androgen receptors [26, 35]. This oestrogenic action of androgens has also been observed in the rat uterus [36], DMBA-induced rat breast tumours [37, 38] and human breast cancers [39]. On the other hand, physiological concentrations of androgens have a negligible stimulatory effect on the growth of MCF-7 cells, except that androst-5-ene-3β,17β-diol is weakly oestrogenic [40]. Growth stimulation via

progesterone or androgen receptor has not been reported in MCF-7 cells, although oestrogens as well as high doses of androgens increase progesterone receptor level via oestrogen receptor [26]. Inhibition of progesterone receptor induction by physiological concentrations of T and DHT may be mediated via the androgen receptor in MCF-7 cells [41]. The stimulation of MCF-7 cell growth by steroid metabolites is therefore considered to be mediated by the oestrogen receptors.

Since the present study is focused on the control mechanisms for intracellular oestrogen levels, events after oestrogen receptor action which result in nuclear DNA synthesis can be excluded. In addition, involvement of various growth factors or second messengers can also be excluded. We therefore used 10% charcoal-treated fetal bovine serum in the culture system to obtain maximum cell viability.

MacIndoe [24] first demonstrated that MCF-7 cells have the ability to catalyse [^{3}H]T into [^{3}H]E_2. He obtained 0.07% conversion into E_2 when the cells in a 75-cm^2 flask were incubated for 6 h with [^{3}H]T at a concentration of 10 nM. The present study has confirmed the presence of aromatase activity in MCF-7 cells using the product isolation method. To obtain the maximal conversion into oestrogens we used [^{3}H]AD in a greater substrate concentration (100 nM) and a greater incubation scale. In our assay conditions, the conversions from [^{3}H]AD to E_1 and E_2 were 0.17 and 0.02%, respectively. Although it is hard to compare directly MacIndoe's data with ours, the aromatase activities determined by the two groups seem to be comparable. The present study has also shown that the aromatase activity of MCF-7 cells can be assayed by the tritiated water method which is convenient and almost as accurate and sensitive as the product isolation method. Our data showed that the aromatase activity of MCF-7 cells is comparable to that of human breast cancer tissue [9–14].

Bradlow [5] calculated that oestrogens derived from *in situ* aromatization was insufficient to saturate oestrogen receptors in breast tumours. Pasqualini *et al.* [22] dmonstrated that in MCF-7 cells, oestrogen 3-sulphates were converted by sulphatase to unconjugated oestrogens, which then stimulated the progesterone receptor concentration. Santner *et al.* [17, 18] studied the kinetic properties of aromatase, sulphatase and 17β-hydroxysteroid dehydrogenase in human breast cancer tissue. They found that the physiological concentration of E_1-S is 10 times higher than those of unconjugated E_1 or E_2 in postmenopausal women [19], and concluded that local production of E_2 from E_1-S through the E_1 sulphatase and 17β-hydroxysteroid dehydrogenase pathway is quantitatively more dominant than that through the aromatase pathway. However, the extent of the stimulation of cell growth by oestrogens produced through the sulphatase or aromatase pathway has not been reported. Moreover, E_1 sulphatase activity in MCF-7 cells is inhibited by dehydroepiandrosterone sulphate [23], the level of which is much higher than that of E_1-S.

In the present study we directly compared the biological activities of androgens and conjugated and unconjugated oestrogens by means of [^{3}H]thymidine incorporation in MCF-7 cells. As shown in Fig. 3, assuming that stimulation of thymidine incorporation by oestrogens and androgens is caused only by the action of E_2 which is produced from each steroid, then $0.8–1.2 \times 10^{-10}$ M E_1 [4], 1×10^{-9} M T and $2–4 \times 10^{-9}$ M AD [42], the postmenopausal circulating levels, produce $1.2–1.6 \times 10^{-11}$ M, 5×10^{-12} M and $3.3–5 \times 10^{-12}$ M E_2, respectively. Therefore, the postmenopausal circulating levels of E_2 ($1–5 \times 10^{-11}$ M) [4] plus E_1 are equivalent to $2.2–6.6 \times 10^{-11}$ M E_2, which stimulates thymidine incorporation to 450–550%. Circulating levels of T plus AD (equivalent to $8.3–10 \times 10^{-12}$ M E_2) stimulate through the aromatase pathway to 280–300%. The E_2 concentration which is produced from T and AD ($8.3–10 \times 10^{-12}$ M) is calculated to be 11–33% of the total intracellular E_2 concentration ($3.0–7.6 \times 10^{-11}$ M), suggesting that E_2 derived from the aromatase pathway accounts for a significant portion of the intracellular concentration. In contrast, $10^{-10}–10^{-9}$ M E_1-S [19], the postmenopausal level, had no effect on stimulation under our conditions. The local concentrations of the steroids may not necessarily equal the circulating level, however, Vermeulen *et al.* [14] measured steroid concentrations in breast tumours by radioimmunoassay, and reported that the tissue level of AD was higher than in plasma but that of E_1-S was lower than in plasma. Reed *et al.* [43] infused [^{3}H]AD and [^{14}C]E_1 into breast cancer patients before operation and found a significant uptake of AD and *in situ* synthesis of E_1. These studies may support a significant contribution of the aromatase pathway. Although oestrogen recep-

tor interaction and aromatase activity and other steroid-metabolizing enzyme activities in breast cancer tissue might be affected by various endogenous factors, the present results suggest that in postmenopausal women, oestrogens produced from circulating levels of T and AD through the aromatase pathway contribute significantly to the total intracellular oestrogen levels. In contrast, our results suggest that the production of active oestrogen from postmenopausal circulating level of E_1-S is small, although the potency is comparable to that reported by Pasqualini *et al.* [22]. This is considerably different from the estimation reported by Santner *et al.* [17, 18]. This may be explained by the difference in methods used for measuring oestrogenic activity (i.e. kinetic studies vs [^{3}H]thymidine incorporation), by a possible variation between the enzyme activity of MCF-7 cells and human breast cancer tissue, or by other coexisting endogenous factors which might affect enzyme activities.

A number of aromatase inhibitors have been developed and their potency and specificity have been studied [44–50]. In the present study we investigated the actions of two inhibitors; a representative competitive inhibitor AG [44, 45], which has been widely used for clinical treatment of metastatic breast cancer patients [3, 4], and a new suicide inhibitor 14α-OHAT [27, 50]. Theoretically irreversible inhibitors are more effective than competitive inhibitors [51, 52], however, these studies have remained focused on kinetic analyses of enzyme inhibition. The inhibition of MCF-7 aromatase catalytic activity by aromatase inhibitors has been demonstrated by MacIndoe *et al.* [53]. In the present study we demonstrated that aromatase inhibitors suppress androgen stimulation of DNA synthesis in MCF-7 cells. This suggests that aromatase inhibitors will suppress the growth of breast cancer not only by lowering the circulating levels of oestrogens which are produced in extragonadal tissues, but also by inhibiting the *in situ* oestrogen production from androgens which are supplied from the circulation. In addition, this finding may support the contention that mechanism-based suicide inhibitors are more effective than competitive inhibitors [51, 52], because suicide inhibitors can irreversibly inactivate aromatase enzymes which are continuously being expressed in cells which are also increasing in number.

Our model would be a useful technique for studying multifactorial regulation of oestrogen-dependent breast cancers. This is also useful, in addition to the kinetic methods, for direct assessment of the biological activities of aromatase inhibitors. Furthermore, this technique would be applicable in estimating the potency of a mechanism-based inhibitor compared with a competitive inhibitor using a simple culture system.

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