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ORIGINAL ARTICLE

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Effects of fadrozole and leuprorelin acetate on aromatase activity and cell proliferation in a human breast cancer cell line (SK-BR-3)

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Abstract
Background. In recent years, aromatase inhibitors have been used to treat hormone-dependent breast cancer in postmenopausal women. Although gonadotropin-releasing hormone (GnRH) agonists inhibit the growth of breast cancers by estrogen deprivation, it is not known whether GnRH agonists have a direct effect on breast cancer cells. In the present study, we examined the direct effect of a GnRH agonist (leuprorelin acetate) on aromatase activity in a human breast cancer cell line, SK-BR-3. We also studied the synergistic effect of fadrozole (an aromatase inhibitor) and leuprorelin acetate on aromatase activity and cell proliferation in SK-BR-3 cells.
Methods. Aromatase activity was determined by measuring [^{3}H] water released upon the conversion of [1β-^{3}H] androstenedione to estrone. Cell proliferation was estimated by determining the incorporation of 5-bromo-2′-deoxyuridine in cellular DNA (cell proliferation assay system).
Results. Aromatase activity in SK-BR-3 was inhibited by fadrozole. In addition, SK-BR-3 aromatase activity was inhibited by leuprorelin acetate. Stimulation of cell proliferation by estradiol (10 nM) and testosterone (20 nM) was almost completely inhibited by the addition of an estrogen receptor antagonist, ICI 182780 (10 nM), and fadrozole (1 nM). When both these compounds were added, the most potent inhibition of aromatase activity (fadrozole, 0.1 nM; leuprorelin acetate, 1 nM) and cell proliferation (fadrozole, 10 nM; leuprorelin acetate, 100 nM) was observed.
Conclusions. These results lead us to the conclusion that combination therapy with an aromatase inhibitor and a GnRH agonist may provide a new treatment for both pre- and postmenopausal patients with hormone-dependent breast cancer.

Key words Fadrozole · Leuprorelin acetate · Aromatase activity · Cell proliferation · SK-BR-3

Introduction

A number of studies have demonstrated that 30% to 60% of breast carcinomas have aromatase activity substantially higher than that in normal breast tissue and surrounding adipose tissues.[1] In postmenopausal women, estrogens produced from adrenal androgens through an aromatase enzyme system (which is located in fat, muscle, and other tissues) play an important role in stimulation of the growth of estrogen-dependent breast cancer.[2] The high concentrations of estradiol found in the breast tumors of postmenopausal women could be the result of enhanced uptake from plasma or in-situ aromatization of androgens to estrogens.[3] As some clinical studies have shown that breast cancers with high aromatase activity are more sensitive to treatment with aromatase inhibitors than tumors without aromatase activity,[4,5] aromatase inhibitors have recently been used for the treatment of hormone-dependent breast cancer in postmenopausal patients.

Gonadotropin-releasing hormone (GnRH) agonists also inhibit the growth of hormone-dependent tumors, such as prostatic and breast cancers. Remission of breast cancer in premenopausal women after GnRH agonist administration is apparently mediated by the suppression of gonadotropin secretion and the resultant decrease in gonadal steroid hormone secretion.[6] Recently, some investigators have reported that GnRH agonist administration, with an estrogen receptor inhibitor, tamoxifen, is an effective treatment for breast cancer in postmenopausal women.[7] In addition, Christian et al.[8] reported the direct effect of a GnRH agonist and tamoxifen in a breast cancer cell line, MCF-7. Although combination therapy of a GnRH agonist with an

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aromatase inhibitor is clinically effective in breast cancer patients,[9,10] few studies have investigated the effect of the combination on breast cancer cells in vitro. In the present study, therefore, we studied the direct effect of a GnRH agonist, leuprorelin acetate, on aromatase activity and cell proliferation in the human breast cancer cell line, SK-BR-3, which is known to have aromatase activity and estrogen and GnRH receptors.[11,12] We also studied the synergistic effect of a potent aromatase inhibitor, fadrozole, and leuprorelin acetate. We also studied the effects of estradiol and testosterone on the proliferation of SK-BR-3 cells.

Materials and methods

Materials

We obtained [1β-^{3}H] androstenedione (SA, 24.7 Ci/mmol), as a substrate for a [^{3}H] water assay, from New England Nuclear (Boston, MA, USA). McCoy's 5A medium and fetal bovine serum (FBS) were purchased from Gibco BRL (Bethesda, MD, USA). A cell proliferation assay system (5-bromo-2-deoxyuridine – enzyme-linked immunosorbent assay; BrdU-ELISA) was obtained from Boehringer Mannheim (Mannheim, Germany). Fadrozole was provided by Novartis Pharmaceuticals (Basel, Switzerland). The GnRH agonist, leuprorelin acetate, was kindly provided by Takeda Chemical Industries (Tokyo, Japan). A competitive estrogen receptor antagonist, ICI 182780, was kindly provided by Zeneca Pharmaceuticals (Macclesfield, England). All other steroids and chemicals were purchased from Sigma (St. Louis, MO, USA).

Cell culture

The human breast adenocarcinoma cell line, SK-BR-3, was obtained from the American Type Culture Collection (Rockville, MD, USA). SK-BR-3 was cultured in McCoy's 5A medium supplemented with penicillin G (40 U/ml), streptomycin (40 μg/ml), and FBS (10%). Cell culture was maintained at 37°C in humidified 5% CO_2-95% air. The medium was changed twice a week. When SK-BR-3 cells reached 80% confluence, the medium was changed to FBS-free medium and the cells were cultured for an additional 48 h.

Aromatase activity

Aromatase activity was measured by the [^{3}H] water method.[13] The medium was changed and cells were incubated with [1β-^{3}H] androstenedione (20 nM; 24.7 Ci/mmol) for an additional 4 h. In some dishes, the aromatase inhibitor, fadrozole, was added simultaneously. When the effect of leuprorelin acetate was determined, cells were pre-incubated with leuprorelin acetate for 48 h.[14] The reaction was terminated by the addition of 0.5 ml trichloroacetic acid (10%). The medium was transferred to a test tube containing 1.5 ml charcoal suspension (30%) to remove residual steroids, and the mixture was incubated at 37°C in water for 30 min. The mixture was centrifuged (3000 *g*) for 10 min and the supernatant was filtered. The radioactivity of [^{3}H] water was measured with a scintilation counter (Aloka LSC-651; Aloka, Tokyo, Japan).

Cell proliferation

Cell proliferation was measured with the BrdU assay system according to the instruction protocol. SK-BR-3 cells, at a density of 5×10^3 cells/well, were dispersed in 96-well culture plates and pre-incubated with FBS-free medium for 48 h. In some dishes, leuprorelin acetate was added. After 48 h, cells were cultured with various concentrations of test substances (fadrozole, ICI 182780, estradiol, or testosterone) for 24 h. BrdU labeling solution was added and the cells were incubated for an additional 18 h. Absorbance was measured with the ELISA reader at 450 nm (reference wavelength, 690 nm).

Statistical analysis

Comparisons between groups were made with the Mann-Whitney *U*-test. Differences were accepted as being significant at $P < 0.05$.

Results

Effect of fadrozole and leuprorelin acetate on aromatase activity in SK-BR-3

SK-BR-3 cells produced [^{3}H] water from [1β-^{3}H] androstenedione during the course of the incubation. The [^{3}H] water release was linear with time for up to 6 h. Thus, in further incubation, the incubation time was set at 4 h. The value for [^{3}H] water radioactivity of the blank incubation without cells was subtracted from the value for each sample. The aromatase activity of the SK-BR-3 cells was calculated as 0.1 pmol/h per 10^6 cells.

Aromatase activity was significantly inhibited ($P < 0.01$) by the addition of fadrozole, in a concentration-dependent manner, with an IC_{50} value of 0.3 nM (Fig. 1A). The administration of 0.1 nM fadrozole inhibited aromatase activity to approximately 60% of the control value, and 1 nM and 10 nM fadrozole inhibited aromatase activity to 20% and 3% of the control value, respectively. The addition of 1 pM leuprorelin acetate inhibited aromatase activity to approximately 80% of the control value, and 1 nM and 1 μM leuprorelin acetate inhibited aromatase activity to 70% and 40% of the control value respectively (Fig. 1B). When both fadrozole (0.1 nM) and leuprorelin acetate (1 nM) were administered, the aromatase activity was almost completely inhibited (Fig. 2).

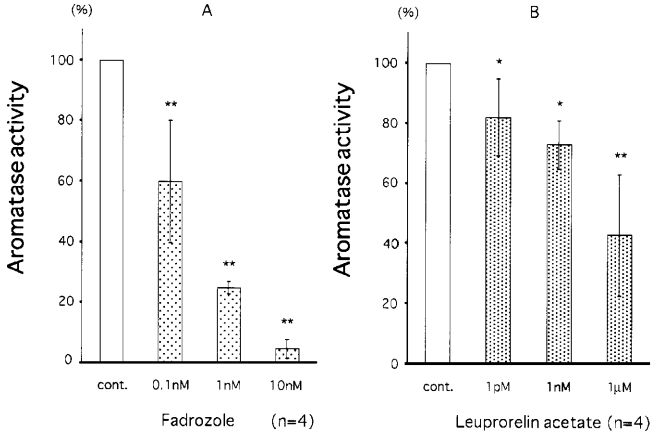

Fig. 1. A Inhibitory effect of fadrozole on aromatase activity in the human breast cancer cell line SK-BR-3. Aromatase activity is expressed as a percentage of the control (*cont.*) activity. Results are expressed as the mean ± SD values from four determinations in two independent experiments. $^{**}P < 0.01$; the effect of fadrozole is significantly different from the value in the absence of fadrozole. **B** Inhibitory effect of leuprorelin acetate on aromatase activity in SK-BR-3. Aromatase activity is expressed as a percentage of the control activity. Control was not added to leuprorelin acetate. Results are expressed as mean ± SD values from four determinations in two independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$, the effect of leuprorelin acetate is significantly different from the value in the absence of leuprorelin acetate

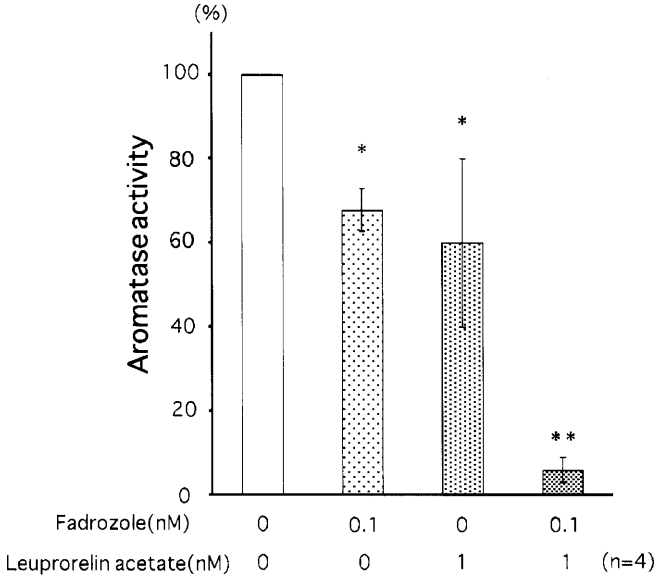

Fig. 2. Effect of the combination of fadrozole and leuprorelin acetate on aromatase activity in SK-BR-3. Aromatase activity is expressed as a percentage of the control activity. Results are expressed as mean ± SD values from four determinations in two independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$, the effect of fadrozole and leuprorelin acetate is significantly different from the value in the absence of fadrozole and leuprorelin acetate

Effect of sex steroids, fadrozole, and leuprorelin acetate on cell proliferation in SK-BR-3

The administration of estradiol at concentrations of 0.1 to 10 nM stimulated cell proliferation significantly ($P < 0.05$),

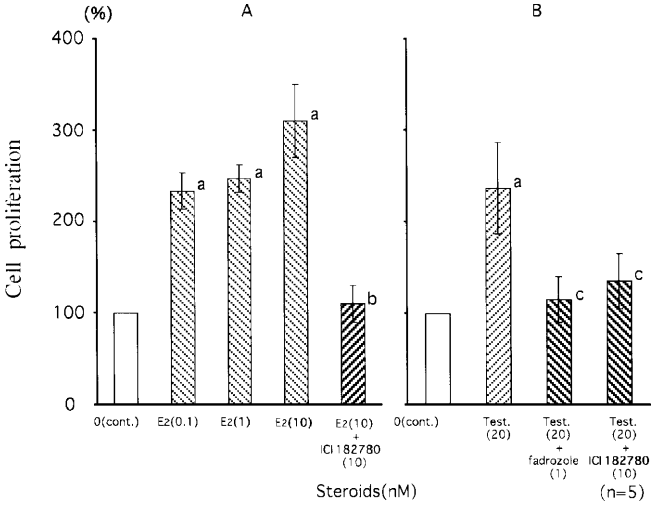

Fig. 3A,B. Effect of steroids, fadrozole, and the competitive estrogen receptor antagonist, ICI 182780, on cell proliferation in SK-BR-3. Cell proliferation is expressed as a percentage of the control count. Results are expressed as mean ± SD values from five determinations. *a*, $P <$ 0.01 vs control; *b*, $P < 0.01$ vs estradiol (*E2*; 10 nm); *c* $P < 0.05$ vs testosterone (*test*; 20 nm)

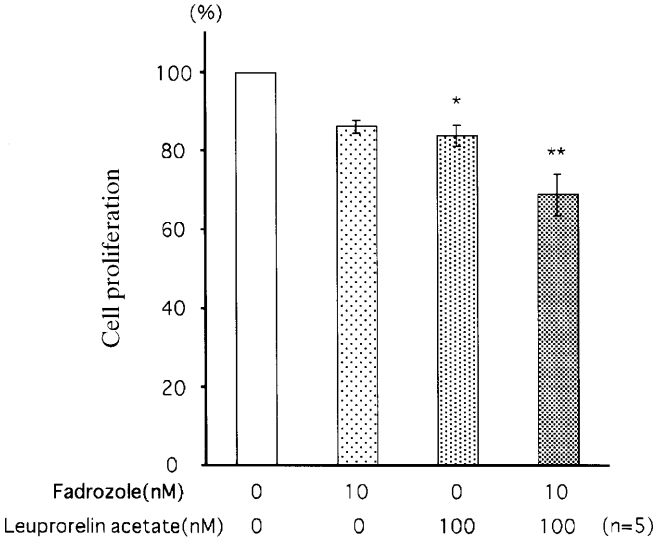

Fig. 4. Effect of the combination of fadrozole and leuprorelin acetate on cell proliferation in SK-BR-3. Cell proliferation is expressed as a percentage of the control count. Results are expressed as mean ± SD values from five determinations. $^{*}P < 0.05$; $^{**}P < 0.01$, the effect of fadrozole and leuprorelin acetate is different from the value in the absence of fadrozole and leuprorelin acetate

in a dose-dependent manner (Fig. 3A). When ICI 182780 (10 nM) was simultaneously added to the medium, the stimulatory effect of estradiol (10 nM) was almost completely inhibited. Testosterone (20 nM) also significantly ($P < 0.05$) stimulated cell proliferation, and the stimulatory effect was similar to that of 0.1 nM estradiol (Fig. 3B). The proliferation stimulated by testosterone (20 nM) was almost completely inhibited by fadrozole (1 nM) and by ICI 182780 (10 nM). As shown in Fig. 4, when 10 nM fadrozole and

100 nM leuprorelin acetate were added to SK-BR-3 cells, the cell proliferation was significantly inhibited ($P < 0.01$), to about 70% of the control value.

Discussion

The present study demonstrated the in-vitro synergistic effect of an aromatase inhibitor, fadrozole, and a GnRH agonist, leuprorelin acetate, on the cell proliferation of SK-BR-3. Both clinical and endocrinological studies have investigated treatment with GnRH agonists in premenopausal breast cancer patients and treatment with aromatase inhibitors in postmenopausal breast cancer patients. The study group of Coombes et al.[9,10] demonstrated that the combination of a GnRH agonist and an aromatase inhibitor given to premenopausal women caused greater estrogen suppression than the GnRH agonist alone, and led to an objective clinical response in four of six patients with breast cancer after the disease relapsed following treatment with a GnRH agonist as a single agent. So far, to our knowledge, the effects of a combination of an aromatase inhibitor and a GnRH agonist on cell proliferation in breast cancer cells have not been reported. Therefore, in the present study, we determined the effects of fadrozole and leuprorelin acetate on aromatase activity and cell proliferation in a human breast cancer cell line.

The human breast cancer cell line SK-BR-3 is known to have aromatase activity and estrogen and GnRH receptors.[11,12] In a preliminary study, we observed the immunohistochemical expression of aromatase in the cytoplasm of SK-BR-3 cells (data not shown). Therefore, we used SK-BR-3 for this present study. Fadrozole significantly ($P < 0.01$) inhibited aromatase activity in SK-BR-3 in a concentration-dependent manner, with an IC_{50} of 0.3 nM. Leuprorelin acetate also significantly ($P < 0.01$) inhibited aromatase activity in SK-BR-3; to our knowledge, this is the first demonstration that leuprorelin had such an inhibitory effect on aromatase activity in a human breast cancer cell line. When 3.75 mg of leuprorelin acetate was administered subcutaneously, to premenopausal breast cancer women, the serum concentration was found to be approximately 0.5 to 1 nM.[14] In the present study, it is interesting to note that 1 nM leuprorelin acetate significantly inhibited aromatase activity, to about 70% of the control value. Maeda et al.[15] reported that a GnRH agonist, buserelin, at concentrations of 0.01 to 1 nM, stimulated aromatase activity in cultured human granulosa cells, while at concentrations of 10 to 100 nM, buserelin suppressed this activity. The difference between their results and ours may reflect the different reagents and cells used. In the present study, fadrozole (0.1 nM) and leuprorelin acetate (1 nM) in SK-BR-3 cells completely inhibited aromatase activity. The inhibitory mechanism of leuprorelin acetate on aromatase activity in SK-BR-3 is still unclear; therefore, further study is necessary to elucidate this question.

Estradiol, at concentrations of 0.1 to 10 nM, showed a stimulatory effect on cell proliferation, in a dose-dependent manner. The addition of 20 nM testosterone to SK-BR-3 cells stimulated cell proliferation, and the stimulatory effect was similar to that of 0.1 nM of estradiol. The cell proliferation stimulated by testosterone (20 nM) was almost completely inhibited by fadrozole (1 nM) and ICI 182780 (10 nM). Yano et al.[16] demonstrated that testosterone-induced cell growth in MCF-7 breast cancer cells was inhibited by fadrozole, but was not inhibited by an antiandrogenic agent. Brian et al.[17] reported that ICI 182780 significantly inhibited the growth of MCF-7 cells. They speculated that the growth inhibition appeared to be caused by the antiestrogenic activity of ICI 182780. Yue et al.[17] reported when ovariectomized nude mice were inoculated with MCF-7 cells transfected with the human placental aromatase gene, the tumor growth was stimulated by the administration of androstenedione. However, when the ovariectomized mice with MCF-7 tumors were treated with the aromatase inhibitors 4-hydroxyandrostenedione or fadrozole, or with the antiestrogen tamoxifen, tumor growth was significantly inhibited. From our data, together with those of previous reports, we assume that androgen was converted into estrogen by intracellular aromatase and stimulated the cell proliferation through estrogen receptors.

When fadrozole (10 nM) and leuprorelin acetate (100 nM) were added to SK-BR-3 cells, their cell proliferation was significantly inhibited. Thus, it is speculated that the inhibition of SK-BR-3 cell proliferation by fadrozole and leuprorelin acetate may be the result of the inhibition of aromatase activity. Concentrations of 0.1 nM fadrozole and 1 nM leuprorelin acetate showed complete inhibition of aromatase activity, while these concentrations did not affect cell proliferation (data not shown). When we measured cell proliferation, we did not add androgen as an aromatase substrate; therefore, the discrepancy in the doses of the agents fad rozole and leuprorelin may be due to a limitation of intrinsic cellular substrates for aromatase.

The remission of breast tumor growth in premenopausal women treated with aromatase inhibitors and GnRH agonists suggested that these peptides may have a direct effect on breast cancer cells.[9,10] Yue et al.[18] reported the first direct evidence that the in-situ synthesis of estrogen in breast tumors, rather than peripheral aromatization and uptake from plasma, can enhance tissue estrogen levels and stimulate tumor growth; GnRH agonists may exert their antitumor activity not only through chemical castration but also by acting directly on tumor cells. Our results lead us to the conclusion that combination therapy with an aromatase inhibitor and a GnRH agonist may provide a new treatment not only for premenopausal but also for postmenopausal women with hormone-dependent breast cancer.

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