Endometrial carcinoma is one of the most common malignancies in developed countries, and its incidence has increased (1). In situ estrogen metabolism, including synthesis and degradation, has recently been thought to play a very important role in the etiology and progression of endometrial carcinoma. Aromatase is a key enzyme in the conversion of androgens to estrogens, and aromatase localization studies have reported that aromatase immunoreactivity and mRNA were detected mainly in stromal cells. However, the effect of tumor-stromal interactions on local estrogen biosynthesis in endometrial carcinomas remains largely unknown.

**Abstract**

**Purpose:** The metabolism and synthesis of intratumoral estrogens are thought to play a very important role in the etiology and progression of endometrial carcinoma. Aromatase is a key enzyme in the conversion of androgens to estrogens, and aromatase localization studies have reported that aromatase immunoreactivity and mRNA were detected mainly in stromal cells. However, the effect of tumor-stromal interactions on local estrogen biosynthesis in endometrial carcinomas remains largely unknown.

**Experimental Design:** The endometrial carcinoma cell lines (Ishikawa and RL95-2) and breast carcinoma cell line (MCF-7) were cocultured with stromal cells isolated from endometrial carcinomas, and aromatization activity was measured using liquid chromatography-tandem mass spectrometry. We then confirmed the local biosynthesis of estrogens and tumor-stromal interactions on aromatase activity in Ishikawa and RL95-2 cells. In addition, we also examined the effects of aromatase inhibitors on cell proliferation.

**Results:** Aromatase activity was significantly higher in cocultures with Ishikawa or RL95-2 than in each monoculture, respectively. Estrone (E1) concentrations were significantly higher than estradiol (E2) concentrations in Ishikawa and RL95-2 cells, whereas E2 was significantly higher than E1 in MCF-7 cells. Cell proliferation was significantly inhibited in Ishikawa and RL95-2 cell cultures treated with aromatase inhibitors compared with control cultures.

**Conclusions:** These results indicate the contribution of not only E2 but also E1 to cancer cell proliferation in endometrial carcinoma. Our study may provide important information on metabolism and synthesis of intratumoral estrogens with regard to the etiology and progression of endometrial carcinoma, thus helping to achieve improved clinical responses in patients with endometrial carcinoma, who are treated with aromatase inhibitors. (Clin Cancer Res 2009;15(19):6028-34)
Translational Relevance

Endometrial carcinoma is estrogen-dependent disease and standard therapy is established as staging laparotomy. However, progesterone is only endocrine therapy for advanced endometrial carcinoma. Therefore, more endocrine treatment is desirable. Estrogens are produced and accumulated by the conversion from androgens through aromatase pathway with tumor-stromal interactions. This is the first study to show the estrogen production using the coculture system. Aromatase activity in coculture was significantly higher than that in monocyte in endometrial carcinoma cells. Interestingly, estrone was significantly higher than estradiol in endometrial carcinoma cells, whereas estradiol was significantly higher than estrone in breast cancer cell line. In addition, we found significant inhibition for cell proliferation treated with aromatase inhibitors. Our study provided important information for the possible treatment in advanced or recurrent endometrial carcinoma patients. We might achieve better clinical responses in endometrial carcinoma patients with aromatase activity through decreasing local estrogen concentration by aromatase inhibitors (tailor-made medicine).

Materials and Methods

Patients and tissue preparations. A total of three endometrial carcinoma specimens were obtained from Japanese patients between 2004 and 2006 at the Department of Obstetrics and Gynecology, Tohoku University Hospital. This study was approved by the Ethical Committee of Tohoku University School of Medicine, and the required informed consents were obtained.

Cells and culture conditions. The human endometrial carcinoma cell line Ishikawa was kindly provided by Dr. Nishioka (Tokyo Medical University Kasumigaura Hospital). The human endometrial carcinoma cell line RL95-2, the breast carcinoma cell line MCF-7, and the mouse fibroblast cell line 3T3-L1 were purchased from the American Type Culture Collection. Ishikawa, RL95-2, MCF-7, and 3T3-L1 cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (BioWest), penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin (250 ng/mL growth medium). Primary stromal cells employed in this study were designated #3, #11, and #16 and were isolated from human endometrial carcinoma tissue samples by collagenase treatment and maintained in RPMI 1640 with 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin as described previously (13). mRNA expression of aromatase and 17β-HSD type 2 in stromal cells were as follows: aromatase (+) and 17β-HSD type 2 (+) in #3 and aromatase (+) and 17β-HSD type 2 (-) in both #11 and #16.

Coculture system. For physical separation of stromal cells and the endometrial carcinoma cell lines, Transwell cultures were established in 6-well plates using Transwell permeable supports (0.4 μm pore; Corning). First, Ishikawa, RL95-2, MCF-7, 3T3-L1, and tumor stromal cells were cultured separately in 100-mm dishes until 70% confluence. Ishikawa, RL95-2, MCF-7, and 3T3-L1 cells were then cultured in Transwell chambers of the 6-well plates in the absence or presence of stromal cells, which were cultivated at the bottom of the 6-well plates. We named this system the coculture system as described previously (12). After 24 h of cultivation in the coculture system, the cell lines and stromal cells were separated again, and each component was examined in the aromatization assay, estrogen production assays, or by real-time quantitative reverse transcription-PCR (qRT-PCR). Cells types after monoculture or coculture were designated with the subscripts MO or CO, respectively (Ishikawamoso, #3co, IshikawaCO, etc.).

Before the assays, viable cells were counted by the trypan blue exclusion method, total RNA was extracted, and mRNA levels were determined by real-time qRT-PCR.

Total RNA extraction and cDNA synthesis. Total RNA was extracted from Ishikawa, RL95-2, MCF-7, 3T3-L1, and stromal cells using the RNeasy Mini Kit (Qiagen). A reverse transcription kit, SuperScript III Platinum CellDirect Two-Step qRT-PCR kit with SYBR Green (Invitrogen), was used for the synthesis of cDNA.

Real-time qRT-PCR. Real-time qRT-PCR was carried out using the LightCycler TaqMan Master Ready-to-Use Hot Start reaction mix for PCR (Roche Diagnostics) for aromatase and the LightCycler FastStart DNA SYBR Green I (Roche Diagnostics) for the other mRNA targets using the LightCycler ST300 instrument (Roche Diagnostics). The primer sequences were as follows: forward 5'-CCCTTTCTGC- GTGTCCTGTATGTC-3' and reverse 5'-GGAGAGGCTTGCCATGCATCAA-3' for aromatase, forward 5'-TGTCAAGGGGCCATGTC-3' and reverse 5'-GGCCGAATTTCTGGTCTGGCGTACCGCATGATTC-3' for estrogen receptor-α (ERα; ref. 14), forward 5'-ACCCAGCAATAGAAGAT-3' and reverse 5'-GCTTAAAGCAAAAGATGGA-3' for β-actin, forward 5'-TGTCTGACGCTGAATGTA-3' and reverse 5'-GTATGGAAGGCGGTGGAAG-3' for 17β-HSD type 1, and forward 5'-CAAGGAAGGCTGTGAAAT-3' and reverse 5'-TACCTCGTCGCTGCTGATA-3' for 17β-HSD type 2. The aromatase primers were designed using the Universal ProbeLibrary System (Roche Diagnostics). β-Actin primers were designed by the Nihon Gene Research Laboratories. 17β-HSD types 1 and 2 primers were type 1 immunoreactivity and mRNA were absent in normal endometrium and hyperplastic endometrium and in endometrial carcinoma (8, 9), and 17β-HSD type 2 expression was detected in normal endometrium (secretory phase) but was decreased in hyperplastic endometrium and endometrial carcinoma (9). This is in contrast to the results of a study of these enzymes in breast cancer, in which nearly half of the cases showed 17β-HSD type 1 immunoreactivity in carcinoma cells, whereas 17β-HSD type 2 was not expressed (10). These reports have been shown that local estrogen biosynthesis was mainly regulated by aromatase and 17β-HSD types 1 and 2 in endometrial carcinoma (Fig. 1).

It is well known that aromatase expression is regulated by various transcriptional factors, including nuclear receptors and their putative ligands, in several types of human cells and tissues (11). However, the correlation between nuclear receptors and aromatase in parenchymal or carcinoma cells of endometrial carcinoma remains largely unknown. To evaluate the potential effects of tumor-stromal interactions on local estrogen biosynthesis in endometrial carcinoma, we established a coculture system using endometrial carcinoma cell lines and stromal cells (12). In the present study, we examine the local biosynthesis of estrogen and the effect of tumor-stromal interactions on aromatase expression and enzyme activity in endometrial carcinoma. In addition, we also investigate the effect of aromatase inhibitors on cell proliferation. In evaluating the metabolism and synthesis of intratumoral estrogens with regard to the etiology and progression of endometrial carcinoma, this study may help improve the way aromatase inhibitors are used to achieve better clinical responses in patients with endometrial carcinoma.

www.aacrjournals.org  6029  Clin Cancer Res 2009;15(19) October 1, 2009

Downloaded from clincancerres.aacrjournals.org on April 15, 2017. © 2009 American Association for Cancer Research.
designed using OLIGO Primer Analysis Software (Takara Bio). The PCR conditions were 45 cycles (95°C for 10 s and 60°C for 35 s) for aromatase, 45 cycles (95°C for 10 s, 65°C for 10 s, and 72°C for 10 s) for β-actin, 45 cycles (95°C for 15 s, 60°C for 5 s, and 72°C for 10 s) for 17β-HSD type 1, and 45 cycles (95°C for 10 s and 70°C for 5 s) for 17β-HSD type 2. To quantify target cDNA transcripts, known concentrations of target gene cDNAs and the β-actin housekeeping gene were used to generate standard curves for real-time qRT-PCR. Each target mRNA level was expressed as the ratio to β-actin mRNA.

Aromatase assay. Aromatase assays were done on IshikawaMO, IshikawaCO, RL95-2MO, RL95-2CO, #16MO, and #16CO with Ishikawa and RL95-2 cells using the 6a-methylandrosten-4-ene-3,17-dione assay to quantify aromatization activity (15). The aromatase conversion of 6a-methylandrosten-4-ene-3,17-dione, an androgen analogue, into an estrogen analogue (6a-methylestradiol) was shown to be highly specific, and an accurate evaluation of aromatase activity could be possible by measuring the production of estrogen analogue (15). 6a-Methylestradiol was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at ASKA Pharma Medical. The activity without cells was used as control.

Estrogen production assay. IshikawaCO, RL95-2CO, and MCF-7CO were incubated at 37°C in fetal bovine serum-free RPMI 1640 containing 10 mmol/L androstenedione (Toronto Research Chemicals) or testosterone (Wako Pure Chemical Industries) as substrate for 24 h. Concentrations of E1 and E2 were evaluated by LC-MS/MS analysis at ASKA Pharma Medical as described previously (16). All the cells treated with substrate were quantified by the trypan blue exclusion method. The concentrations without cells were regarded as control.

Cell proliferation assay. After coculture with stromal cells for 24 h, Ishikawa and RL95-2 cells were trypsinized, harvested, and seeded in phenol red–free and fetal bovine serum–free medium with 10−8 mol/L testosterone in 96-well plates (5 × 104 cells/mL) for 24 h. We then added additional (10−8 mol/L) testosterone and aromatase inhibitors (10−6-10−10 mol/L), which were either steroidal aromatase inhibitor, anastrozole (Toronto Research Chemicals), or nonsteroidal aromatase inhibitor, exemestane (LKT Laboratories). Ethanol was used as the vehicle. Cell proliferation was evaluated using the WST-8 method (Cell Counting Kit-8; Dojindo Molecular Technologies) at 0, 24, 48, and 72 h.

Statistical analysis. Data were analyzed using the t test for evaluation of two groups, employing the StatFlex 5.0 software program (Artec).

Results

Expression of aromatase, 17β-HSD types 1 and 2, and ERα mRNAs in Ishikawa, RL95-2, and MCF-7 cell cultures. The mRNA expression levels of 17β-HSD types 1 and 2, aromatase, and ERα were determined in Ishikawa, RL95-2, and MCF-7 (Fig. 2A-D) cell lines. For all cell lines, no significant differences were seen in mRNA expression levels for the enzymes and ERα between monoculture and coculture. 17β-HSD type 1 and 2 expression and aromatization expression were shown in all cell lines. ERα expression was also expressed in all cell lines.

Detection of aromatase activity in Ishikawa and RL95-2 cells. To estimate the interaction between cancer and stromal cells, aromatization activity in the coculture system was measured by LC-MS/MS. Our results showed that IshikawaMO, RL95-2MO, and #16MO had aromatase activity. Aromatase activity was significantly higher in IshikawaMO, RL95-2CO, and #16CO compared with IshikawaMO, RL95-2MO, and #16MO, respectively (P < 0.05; Fig. 3).

Concentrations of estrogen with androgen as the substrate. Estrogen production in Ishikawa, RL95-2, and MCF-7 was measured using androstenedione or testosterone as the substrate by LC-MS/MS. Both E1 and E2 were produced in monoculture and coculture systems. E1 levels of coculture were higher than those of monoculture in Ishikawa and RL95-2 and E2 levels of coculture were higher than those of monoculture in MCF-7 (data not shown). In the case of androstenedione as the substrate, E1 levels were significantly higher than E2 in Ishikawa and RL95-2 (P < 0.001 and P < 0.001, respectively; Fig. 4A).
The results indicate that androstenedione was mainly converted to E1 by aromatase in Ishikawa and RL95-2 cells. In MCF-7, higher conversion of E1 to E2 by 17β-HSD type 1 resulted in a high E2 level. In the case of testosterone as the substrate, E1 levels were higher than E2 in Ishikawa and RL95-2 (\( P < 0.01 \)), whereas E2 levels were significantly higher than E1 in MCF-7 (\( P < 0.01 \); Fig. 4B). The results indicate that testosterone was converted to E2 by aromatase in Ishikawa and RL95-2 cells and stromal cells, and E2 was converted to E1 by 17β-HSD type 2. In MCF-7, lower conversion of E2 to E1 by 17β-HSD type 2 resulted in a high E2 level. In addition, estrogen levels were markedly decreased by the aromatase inhibitors anastrozole and exemestane (data not shown).

Effect of aromatase inhibitors on cell proliferation. To estimate the inhibition of endometrial carcinoma growth by aromatase inhibitors, Ishikawa and RL95-2 cell proliferation assays were done. Before the appropriate cell proliferation inhibitory concentrations of aromatase inhibitors were determined, Ishikawa, RL95-2, and #16 stromal cell cocultures were treated with aromatase inhibitor concentrations from \( 10^{-6} \) to \( 10^{-10} \) mol/L using ethanol as the vehicle. Cell proliferation was significantly inhibited in both Ishikawa and RL95-2 cells treated with any concentration of anastrozole and exemestane compared with the vehicle control. We found significant differences in cell proliferation between the treatment with \( 10^{-6} \) to \( 10^{-8} \) mol/L and the \( 10^{-9} \) to \( 10^{-10} \) mol/L anastrozole and exemestane (data not shown). We reported previously that \( 10^{-8} \) mol/L aromatase inhibitors clearly inhibited the aromatase activity in MCF-7 (12). Therefore, we determined the appropriate...
concentration of aromatase inhibitors as 10^{-8} \text{ mol/L}, which was effective and less toxic. We employed six experimental coculture systems treated with 10^{-8} \text{ mol/L} aromatase inhibitors, which included Ishikawa plus #3, #11, or #16 stromal cells and RL95-2 plus #3, #11, or #16 stromal cells. In addition, 3T3-L1 cells cocultured with stromal cells #3, #11, or #16 were used as controls because those cells do not express aromatase. We found significant differences in cell proliferation between any IshikawaCO treated with 10^{-8} \text{ mol/L} aromatase inhibitors and controls (Fig. 5A and B). We also found the same results between any RL95-2CO treated with 10^{-8} \text{ mol/L} aromatase inhibitors and controls (Fig. 5C and D). There was no significant inhibition of cell proliferation in 3T3-L1 CO treated with 10^{-8} \text{ mol/L} anastrozole or exemestane.

To evaluate the effect of 17\beta-HSD type 2 on cell growth, cell proliferation rates were analyzed comparatively in the coculture system of Ishikawa and RL95-2 plus #3, #11, or #16 stromal cells, which had different expression patterns of 17\beta-HSD type 2. However, there were no significant differences seen in cell proliferation, although 17\beta-HSD type 2 expression varied (data not shown).

**Discussion**

The estrogens, especially E_2, which is a biologically potent estrogen, have been shown to contribute greatly to the growth and the development of estrogen-dependent tumors in endometrial carcinomas and breast carcinomas (17, 18). However, it is also true that the great majority of endometrial carcinomas occur during the postmenopausal period, when the ovaries no longer produce active sex steroids and circulating plasma estrogens are at very low concentrations. Numerous studies have reported that there was no consistent evidence of increased serum estrogen concentrations or other systemic estrogen abnormalities in women with endometrial carcinoma (19, 20). Tseng et al. and Yamaki et al. reported on the conversion of androgens to estrogens through aromatase in endometrial carcinoma (21, 22). In addition, Watanabe et al. have reported that aromatase protein and mRNA expression were predominantly detected in stromal cells (5).

In this study, we employed a coculture system that could provide important information regarding the intratumoral microenvironment, such as evaluation of cell-cell interactions (23). Aromatase activities in Ishikawa and RL95-2 cocultures were significantly higher than activities in each monoculture. The results indicate that aromatase activity was increased by tumor-stromal interactions. It has been reported previously that various aromatase-stimulating factors, such as interleukin-1, interleukin-6, interleukin-11, tumor necrosis factor-\alpha, prostaglandin E_2, etc., are released from stromal or carcinoma cells in human breast carcinoma (24–26). However, in endometrial carcinoma, the possible effects of aromatase-stimulating factors

**Fig. 3.** Aromatase activity in IshikawaCO and RL95-2CO with #16 stromal cells was significantly higher than that in IshikawaMO and RL95-2MO. Aromatase assay was done for IshikawaMO, IshikawaCO, RL95-2MO, RL95-2CO, #16MO, and #16CO using 6a-methylandrost-4-ene-3,17-dione. The activity was measured by LC-MS/MS method. Mean ± SE. *, P < 0.01 versus RL95-2MO; **, P < 0.001 versus IshikawaMO and versus #16MO.

**Fig. 4.** Ratio of E_1/E_2, which was produced by aromatase, was contrary between Ishikawa, RL95-2, and MCF-7. Estrogen production assays were done treating with androstenedione (A) and testosterone (B) as substrate. We employed coculture system using Ishikawa, RL95-2, and MCF-7 with #16 stromal cells. Concentrations of E_1 and E_2 were measured by LC-MS/MS method. The buffer with androgen as substrate was regarded as control. Mean ± SE. **, P < 0.01 versus E_2; +, P < 0.001 versus E_2.
secreted from stromal cells on aromatase expression in stromal or carcinoma cells remain largely unknown. Further investigation is needed to elucidate their role in endometrial carcinoma. It has been reported that intratumoral estrogen metabolism is different between endometrial carcinoma and breast carcinoma, although both of them are estrogen-dependent malignancies (27). 17β-HSD types 1 and 2 catalyze the reversible conversion of $E_1$ and $E_2$. 17β-HSD type 1 catalyzes the 17β-reduction of $E_1$ to $E_2$ (6), whereas 17β-HSD type 2 catalyzes the oxidation of $E_2$ to $E_1$ (7). It has been reported that 17β-HSD type 1 regulates the tissue concentrations of $E_2$ in breast carcinoma (28), and we have shown previously that 17β-HSD type 2 mainly regulates the tissue concentrations of $E_2$ and modulates estrogenic actions in normal endometrium and endometrial carcinoma (9).

This is the first study to measure aromatase activity and the amounts of estrogen production using coculture system and LC-MS/MS method in endometrial carcinoma cells. Previous studies have reported that androgen was converted into estrogen through aromatase in normal human endometrium and endometrial carcinoma tissues (21, 22). However, the aromatase activity and the amounts of estrogen production were

![Graphs and charts showing cell proliferation inhibition by aromatase inhibitors.](https://example.com/fig5.png)

**Fig. 5.** Cell proliferation was significantly inhibited by aromatase inhibitors regardless of 17β-HSD type 2 expression. Cell proliferations of IshikawaCO and RL95-2CO following treatment with 10⁻⁸ mol/L anastrozole (Ana) and exemestane (Exe) aromatase inhibitors. Cell proliferation was evaluated using WST-8 method. Mean ± SE. ++, $P < 0.0001$ versus control; +, $P < 0.001$ versus control; **, $P < 0.01$ versus control; *, $P < 0.05$ versus control; ***, $P < 0.005$ versus control; ****, $P < 0.0005$ versus control. A, IshikawaCO cell proliferation with anastrozole. B, IshikawaCO cell proliferation with exemestane. C, RL95-2CO cell proliferation with anastrozole. D, RL95-2CO cell proliferation with exemestane.
not measured in detail. Our results to measure those values confirmed their reports on androgen conversion to estrogen through aromatase. In addition, we also measured the concentrations of E1 and E2 by LC-MS/MS. Interestingly, E1 was significantly higher than E2 in Ishikawa and RL95-2, whereas E2 was significantly higher than E1 in MCF-7. The results indicate that E2 produced by aromatase was converted into E1 by 17β-HSD type 2 and E2 produced by aromatase was not converted into E2 by low 17β-HSD type 1 in Ishikawa and RL95-2 cells but not in MCF-7 cells.

This study confirmed the importance of aromatase to estrogen metabolism and synthesis in endometrial carcinoma. The therapeutic use of aromatase inhibitors has been well defined for breast cancer. However, the therapeutic value of aromatase inhibitors to endometrial carcinoma is not clear. Some reports on the use of aromatase inhibitors for endometrial carcinoma have shown small or minimal effects, and these results were not dramatic and remain controversial (29, 30). However, those patients treated with aromatase inhibitor have not been checked for aromatase expression. To examine the possible effects of aromatase inhibitors on endometrial carcinoma, we first determined the expressions of aromatase and 17β-HSD type 2 in tumor stromal cells derived from several patients.

Then we examined the effects of aromatase inhibitors on cell proliferation in Ishikawa and RL95-2 cocultures, in which the expression of aromatase and 17β-HSD type 2 varied. We found significant inhibition of cell proliferation using anastrozole and exemestane whether 17β-HSD type 2 was expressed or not. E2 is biologically regarded as potential estrogen and we have reported that 17β-HSD type 2 might play some protective and/or suppressive roles against unopposed estrogenic effects by decreasing local estrogen activity (9). However, our results indicate the roles of not only E2 but also E1 in cancer proliferation.

In conclusion, we showed estrogen production through the aromatase pathway using androgen as substrate in the coculture system. Our study may provide important information on metabolism and synthesis of intratumoral estrogens with regard to the etiology and progression of endometrial carcinoma, thus helping to achieve improved clinical responses in patients with endometrial carcinoma, who are treated with aromatase inhibitors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

Local Biosynthesis of Estrogen in Human Endometrial Carcinoma through Tumor-Stromal Cell Interactions
Naomi Takahashi-Shiga, Hiroki Utsunomiya, Yasuhiro Miki, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-09-1013

Cited articles
This article cites 30 articles, 9 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/15/19/6028.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/15/19/6028.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.