Local Biosynthesis of Estrogen in Human Endometrial Carcinoma through Tumor-Stromal Cell Interactions

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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) 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)

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 development and progression of various human estrogen-dependent neoplasms. The results of several studies have shown that the concentration of estradiol (E2) in endometrial carcinoma tissue was significantly higher than the concentration in normal endometrium (2). Our results from a previous study were generally consistent with those of other investigations; we found that E2, estrone (E1), and testosterone levels in tumor tissues were several times higher than concentrations measured in serum (3). These findings indicate that intratumoral estrogen metabolism and synthesis is important in the etiology and progression of endometrial carcinoma.

Intratumoral production of estrogen occurs as a result of the aromatization of androgens such as androstenedione and testosterone into estrogens, and it is catalyzed by the cytochrome P450 aromatase enzyme (4). An aromatase localization study has shown that aromatase immunoreactivity and mRNA were detected mainly in the stromal cells or fibroblasts of endometrial carcinoma but not in normal epithelium (5). The reversible conversions of E1 and E2 are catalyzed by 17β-hydroxysteroid dehydrogenase (17β-HSD) types 1 and 2. The 17β-reduction of biologically less active E1 is catalyzed to E2 by 17β-HSD type 1 (6), and the oxidation of E2 to E1 is catalyzed by 17β-HSD type 2 (7). It was reported that 17β-HSD
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Translational Relevance

Endometrial carcinoma is estrogen-dependent disease and standard therapy is established as staging laparotomy. However, progestin is only endocrine therapy for advanced endometrial carcinoma. Therefore, a 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 monoculture 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. Nishida (Tokyo Medical University Kasumigaura Hospital). The human endometrial carcinoma cell line RL95-2, the breast cancer 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 expressions of aromatase and 17β-HSD type 2 in stromal cells were as follows: aromatase (+) and 17β-HSD type 2 (+) in #3 and aromatase (+) and 17α-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 (IshikawaCO, #3CO, Ishikawa3CO, 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 Direct 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 Master 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′-CCCTCTGCT-GTCCGTGTCACTGCT-3′ and reverse 5′-GGGAGACCTTGGCCATGCA-3′ for aromatase, forward 5′-TGTCAAAAGAGGTGC-3′ and reverse 5′-GGCGCAGAATTTCTCTTGC-3′ for 17β-HSD type 1, and forward 5′-CAAAGGGAGGTCCATGCA-3′ and reverse 5′-CAAGCTTGGGCTGCTGCTG-3′ for 17β-HSD type 2. The primer sequences 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...
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 ERα, 35 cycles (95°C for 10 s, 63°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-methylandrost-4-ene-3,17-dione assay to quantify aromatization activity (15). The aromatase conversion of 6a-methylandrost-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 mmol/L testosterone in 96-well plates (5 × 10⁴ cells/mL) for 24 h. We then added additional (10⁻⁶–10⁻¹⁰ mmol/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.

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 types 1 and 2 and aromatase 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 IshikawaCO, RL95-2MO, and #16MO, respectively (P < 0.05, P < 0.01, and P < 0.05; Fig. 3).

Concentrations of estrogen with androgen as the substrate. Estrone production in Ishikawa and 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 were significantly 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), whereas E2 levels were significantly higher than E1 in MCF-7 (P < 0.001; Fig. 4A).

*Figure 1. Sketch of intratumoral estrogens metabolism and synthesis in endometrial carcinoma. Androgens as substrate are supplied from the blood. Androstenedione and testosterone are converted into E1 and E2 by aromatase mainly in stromal cells, respectively. 17β-HSD types 1 and 2 catalyze the reversible conversion of E1 and E2. The local regulation of estrogen activity is quite different between endometrial carcinoma and breast carcinoma. 17β-HSD type 1 was absent in endometrial carcinoma. 17β-HSD type 2 was expressed in secretary phase but decreased in endometrial carcinoma.*

*Figure 2. Detection of aromatase activity in Ishikawa and RL95-2 cells. Aromatase assays were done on IshikawaMO, IshikawaCO, RL95-2MO, RL95-2CO, #16MO, and #16CO, with Ishikawa and RL95-2 cells using the 6a-methylandrost-4-ene-3,17-dione assay to quantify aromatization activity (15). The aromatase conversion of 6a-methylandrost-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.*
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 and nonsignificant), 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 in a dose-dependent manner 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 $10^{-8}$ mol/L, which was effective and less toxic. We employed six experimental coculture systems treated with $10^{-8}$ 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 Ishikawa CO treated with $10^{-8}$ mol/L aromatase inhibitors and controls (Fig. 5A and B). We also found the same results between any RL95-2 CO treated with $10^{-8}$ mol/L aromatase inhibitors and controls (Fig. 5C and D). There was no significant inhibition of cell proliferation in 3T3-L1 CO treated with $10^{-8}$ mol/L anastrozole or exemestane.

To evaluate the effect of 17β-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 17β-HSD type 2. However, there were no significant differences seen in cell proliferation, although 17β-HSD type 2 expression varied (data not shown).

**Discussion**

The estrogens, especially E2, 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-α, prostaglandin E2, 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 on estrogen production were not investigated. In this study, we employed coculture system using Ishikawa, RL95-2, and MCF-7 with #16 stromal cells. Concentrations of E1 and E2 were measured by LC-MS/MS method. The buffer with androgen as substrate was regarded as control. Mean ± SE. **, $P < 0.01$ versus E2; *, $P < 0.001$ versus E2.

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

**Fig. 4.** Ratio of E1/E2, 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 E1 and E2 were measured by LC-MS/MS method. The buffer with androgen as substrate was regarded as control. Mean ± SE. **, $P < 0.01$ versus E2; +, $P < 0.001$ versus E2.
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 E₁ and E₂. 17β-HSD type 1 catalyzes the 17β-reduction of E₁ to E₂ (6), whereas 17β-HSD type 2 catalyzes the oxidation of E₂ to E₁ (7). It has been reported that 17β-HSD type 1 regulates the tissue concentrations of E₂ in breast carcinoma (28), and we have shown previously that 17β-HSD type 2 mainly regulates the tissue concentrations of E₂ 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...
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 E1 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, these 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 whatever 17β-HSD type 2 was expressed or not. E2 is biologically regarded as potent 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.

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