Induction of Aromatase Expression in Cervical Carcinomas: Effects of Endogenous Estrogen on Cervical Cancer Cell Proliferation

Hareesh B. Nair, Roopa Luthra, Namee Kirmi, Ya-Guang Liu, Lisa Flowers, Dean Evans, and Rajeshwar Rao Tekmal

Abstract

Epidemiologic studies have implicated estrogenic exposure as well as human papilloma virus (HPV) infection in cervical carcinogenesis, and some studies have suggested that estrogen and HPV may play synergistic roles in cervical tumorigenesis. In this study, we report a novel finding that ~35% of cervical carcinomas tested (n = 19) express aromatase, the enzyme responsible for converting androgen to estrogen, the rate-limiting and final step in estrogen biosynthesis. On the other hand, no aromatase expression was detected in precancerous (n = 42) or normal cervical (n = 17) tissue samples. Increased aromatase was associated with increases in estrogen receptors (ER-α and ER-β) and a decrease in progesterone receptor levels, suggesting that in situ estrogen signaling via ER may be involved in tumor growth. Stable overexpression of aromatase in HPV+ cervical cancer cells resulted in increased cellular proliferation, anchorage-independent growth, and ER expression and activity. In contrast, little change in ER was observed in HPV− cells. Steroid hormone receptor expression observed in vitro paralleled that seen in cervical carcinomas expressing aromatase. Aromatase overexpression also induced the expression of cyclin D1, proliferating cell nuclear antigen, and the HPV oncoproteins, E6 and E7. Furthermore, the data underscores the importance of steroid receptor (estrogen and progesterone receptors) regulation in cervical carcinogenesis. To our knowledge, this is the first report demonstrating the induction of aromatase expression in cervical carcinomas, and opens the possibility that aromatase inhibitors may be potential therapeutic agents in cervical carcinomas expressing aromatase. (Cancer Res 2005; 65(23): 11164-73)

Introduction

Cervical cancer is the third leading cause of cancer death in women. Over a half a million new cases are diagnosed every year all over the world. The most common histologic type of cervical carcinoma is squamous cell carcinoma which accounts for >80% of all cervical cancers (1). Although cervical cancer cases are more prevalent in developing countries, there is an increasing trend of mortality due to cervical cancer every year in developed countries.

An increasing number of reports support the association between human papilloma virus (HPV) infection and cervical carcinogenesis (2–5). On the other hand, not all women infected with HPV develop cervical cancer, indicating that other factors are involved along with HPV to induce cervical carcinogenesis. Sex hormones have been shown to increase gene expression of HPV 16 and HPV 18, the two HPV subtypes most associated with cervical cancer (6–10). In addition, most cases of cervical cancer arise in the transformation zone, the most estrogen-sensitive region of the cervix (11). Furthermore, studies suggest that DNA damage by estrogen metabolites may be a factor in cervical carcinogenesis (11, 12).

Several studies have shown a link between hormonal exposure and risk of cervical cancer (13). One study has shown that women taking oral contraceptives are at an increased risk of developing cervical cancer, and that their carcinomas possessed higher levels of estrogen receptor (ER) than women who have not taken estrogen-containing oral contraceptives (14, 15). Parity has also been shown to be a cofactor along with HPV in increased risk of developing cervical cancer (16). Furthermore, women whose mothers were prescribed the synthetic estrogen diethylstilbestrol during pregnancy are at high risk of developing cervicovaginal adenosis and cervical adenocarcinomas (17, 18). Similar lesions were observed in mice that were exposed to diethylstilbestrol perinatally (19–21). A recent study with ER-α knockout mice perinatally treated with diethylstilbestrol has found that ER-α is required for the development of cervicovaginal adenosin (22).

Aromatase is the enzyme responsible for the conversion of androgens to estrogens. Although aromatase expression occurs mainly in the ovaries, other tissues have been shown to express this enzyme. Overexpression of aromatase, a potential etiologic factor in hormone-sensitive cancers, is known to increase estrogenic activity in breast tissue and is associated with various precancerous lesions due to deregulation of steroid receptors, growth, and cell cycle factors (13–16, 23–25). Furthermore, increased aromatase expression has been detected in postmenopausal breast cancers, attesting to the involvement of local estrogen synthesis in breast tumorigenesis (25, 26).

Recent studies by Arbeit et al. showed direct hormonal activation of viral genome in K14 promoter-HPV transgenic mice, in which exogenous estrogen exposure induced multistage neoplastic progression in the squamous epithelium of cervix and vagina in 100% of transgenic mice (26). Thus, it seems that estrogen contributes to HPV persistence and subsequent neoplastic progression by increasing viral gene expression. In this study, our aim was to examine whether local aromatase expression plays a role in cervical cancer.

We have examined the expression of aromatase in cervical cancers of varying stages, dysplastic lesions, and normal cervical tissue. In addition, we have studied the effects of aromatase overexpression in cervical cancer cell lines.

Note: H. R. Nair and R. Luthra contributed equally to this work.

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To the best of our knowledge, this is the first study reporting in situ expression of aromatase in cervical cancer. The data revealed that ~35% of the cervical cancers tested expressed aromatase in situ, and that increased ER levels and decreased progesterone receptor (PR) levels may enhance hormone responsiveness and abolish PR-protective effects, respectively.

Materials and Methods

Tissues and clinical samples. Informed consent, as well as a detailed historical information questionnaire, was obtained from each patient. Samples from cervical cancer and benign cases (women who were having a hysterectomy for benign reasons) were collected from patients operated on at Grady Hospital (Atlanta, GA), Emory University (Atlanta, GA), and Crawford Long Hospital (Atlanta, GA), all associated with the Emory University School of Medicine. The samples were immediately frozen and stored at −70°C for future analyses. All procedures and tissue collection were approved by the Institutional Review Board.

Cell culture and reagents. Cervical carcinoma cell lines, CaSkii, C33A, C4I, and HT3, were obtained from American Type Culture Collection (Rockville, MD) and were cultured in growth medium recommended by the American Type Culture Collection. Reagents for cell culture were purchased from Cellgro (Herndon, VA). 4-Androstene-3,17-dione was purchased from Sigma-Aldrich (St. Louis, MO). Letrozole was synthesized in laboratories of Novartis Pharma AG (Basel, Switzerland) and ICI 182,780 was purchased from Tocris (Ellisville, MO).

RNA analysis. Total RNA from cervical tissues and cervical cancer cells was isolated using TRI reagent according to the manufacturer’s protocol (Sigma-Aldrich). Expression of various steroid receptors and other genes was studied by RT-PCR, using Gene Amp RNA PCR kit (Perkin-Elmer, Foster City, CA). 0.25 μg of mRNA species, 100 ng to 1 μg of total RNA was used as a starting template in the reverse transcription reaction mix.

Amplification of target genes was studied using specific primers as shown in Table 1. PCR products were visualized by agarose gel electrophoresis followed by ethidium bromide staining. Densitometric analysis of RT-PCR products was used for quantifying the differences in the expression of mRNA levels in different samples. The data were normalized to the expression of β-actin, an invariant housekeeping gene. For real-time PCR, a total of 25 μL reaction mix was prepared containing MgCl2 (2 mmol/L), 12.5 μL of 2 × Taq PCR Master mix (Qiagen, Valencia, CA), 0.25 × Syber Green dye (Fisher Scientific, Pittsburgh, PA) and specific primer sets (0.3 μmol/L each). The PCR reaction was set for 40 cycles and the data was compared after normalization with β-actin RNA levels.

Protein analysis. Expression of various proteins was carried out using Western blot analysis. Total protein was isolated from the cervical tissues by homogenization in lysis buffer. Equal amount of protein (60 μg) from representative samples were separated on a denaturing polyacrylamide gel and transferred to a nylon membrane. Non-specific binding of antibodies was blocked by incubation (overnight, 4 °C) in TBS containing 0.1% Triton X-100 (TBST) and 5% nonfat dry milk. Membranes were then incubated with respective primary antibodies in TBST and 5% milk overnight at 4 °C. Specific binding to target protein was visualized by using species-specific IgG followed by chemiluminescent detection and exposure to enhanced chemiluminescence hyperfilm (Amersham, Piscataway, NJ). Antibodies for Western analysis were purchased from NeoMarkers (Fremont, CA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Stable transfection. Human aromatase cDNA expression vector PH-A (a gift from Dr. Shuian Chen, Beckman Institute, City of Hope, CA) was transfected into cervical cancer cell lines (CaSkii, HT-3, C4I, and C33A) using FuGENE 6 (Roche, Indianapolis, IN) according to the manufacturer’s protocol. The cells were maintained in regular medium with 400 μg of G418 Sulfate (Cellgro). Once visible colonies were formed, they were picked using cloning rings and cultured separately to generate stable clones.

Table 1. Primer sets used for RT-PCR amplifications

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>Aromatase</td>
<td>AGCATGCTGTACACGACCCTGT (S)</td>
</tr>
<tr>
<td></td>
<td>TCATCATCACACTGGCCATGT (AS)</td>
</tr>
<tr>
<td>ERα</td>
<td>TGTGCAATGACTATGCTTCA (S)</td>
</tr>
<tr>
<td></td>
<td>GCTCTTCTTCTGTTTITTAA (AS)</td>
</tr>
<tr>
<td>ERβ</td>
<td>GTCCATGCGCTTATCATACATC (S)</td>
</tr>
<tr>
<td></td>
<td>GCTTATCATCTTCCACAGCA (AS)</td>
</tr>
<tr>
<td>PR</td>
<td>ACAGAATCTGATGGCCTGTTGTCGACG (S)</td>
</tr>
<tr>
<td></td>
<td>GCAGCGATGCTTCCTTCTTTCAT (AS)</td>
</tr>
<tr>
<td>VEGF</td>
<td>TGCTTCTACCTCCACATGGCAAGT (S)</td>
</tr>
<tr>
<td></td>
<td>GCGCAGATGCTTCCTTCTTTCAT (AS)</td>
</tr>
<tr>
<td>HPV-E6</td>
<td>CTTCTGAATGCGACCATGACCCACAAGAGAAG</td>
</tr>
<tr>
<td></td>
<td>CTGCA (S)</td>
</tr>
<tr>
<td>HPV-E7</td>
<td>CCCTGAGATGATCTCCTGATCGATACCA (AS)</td>
</tr>
<tr>
<td></td>
<td>CTTGATATAGCCACCATGACCGAGATACAA (AS)</td>
</tr>
<tr>
<td></td>
<td>CCTACA (S)</td>
</tr>
<tr>
<td></td>
<td>CCCTCGAGATGACCCATGTACATTAGGG (AS)</td>
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Aromatase activity assay. To measure aromatase enzymatic activity, we used the tritiated water-release assay, using [3H]androstenedione as the substrate (27). Cells grown in phenol-free medium/charcoal-stripped fetal bovine serum were suspended in assay mixture containing 0.1% bovine serum albumin, 67 mmol/L KPO4 (pH 7.4), and 2.0 μmol/L progesterone. After sonication, 100 nmol/L of [3H]Androstenedione (25.3 Ci/mmol, NEN-962; Perkin-Elmer) was added and the mixture was incubated for 10 minutes at room temperature. NADPH was then added to a final concentration of 1.2 mmol/L, followed by 37 °C incubation and the addition of an equal volume of 3% trichloroacetic acid. Supernatant was collected and extracted with an equal volume of chloroform. Dextran-coated charcoal was added to the assay mixture, which was then vortexed and centrifuged. The supernatant was then added to scintillation fluid and measured in a scintillation counter.

Proliferation assay. To study the effect of endogenous estrogen on cell proliferation, we used the American Type Culture Collection (Manassas, VA) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay kit according to the manufacturer’s directions. A total of 0.3 × 104 cells/ml were seeded, and after overnight incubation, the medium was replaced with 10% charcoal-striped fetal bovine serum. Androstenedione (100 nmol/L) and Letrozole (1 μmol/L) were added to the corresponding wells. After 24 hours, cells were assayed for proliferation.

Anchorage-independent assay. To determine the effects of endogenous estrogen as a result of aromatase expression on malignant growth of cervical cancer cells, we did anchorage-independent growth assay as described previously (28). Cells were seeded at equivalent densities in the presence of aromatase substrate androstenedione (100 nmol/L). The colony size and number of colonies formed was scored 4 weeks after plating.

Transient transfection and luciferase reporter gene assay. One microgram of estrogen response element (ERE)-luc vector (a gift from Dr. Ratna Vadlamudi, University of Texas, M.D. Anderson Cancer Center, Houston, TX) and 10 ng of Renilla luciferase reporter control vector.
(pRL-TK, Promega, Madison, WI) were transiently cotransfected to measure ER activation and transcription efficiency, respectively. Cells were seeded overnight at a density of 5 × 10^5 cells per well in six-well plates before transfection. Cells were transfected using FuGENE 6 transfection reagent (Roche) according to the manufacturer’s protocol to determine ER/ERE activity. Transfection was followed by incubation in charcoal-stripped medium without phenol red for 48 hours and then lysed in passive lysis buffer (Promega) and aliquots of the lysate were used for the assay of luciferase activity using a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA), and was expressed in relative light units. Luciferase activity was then normalized to that of Renilla luciferase.

Inhibition of estrogen receptor-α expression using receptor-specific small interfering RNA molecules. For small interfering RNA (siRNA) studies, a smart pool of double-stranded ER-siRNA (siRNA and siAB kit) as well as nonspecific siRNA were obtained from Dharmacon (Lafayette, CO). Transfection was done with 50 nmol of ER-siRNA and FuGENE 6 reagent according to the manufacturer’s instructions.

Statistical significance. Student’s t test was used to determine the statistical difference in cell proliferation and ERE-luciferase reporter activity between control and experimental groups. P < 0.01 was considered to be statistically significant.

Results

Aromatase is expressed in cervical carcinoma but not in normal cervix. It is known that the cervix is an estrogen-responsive tissue and that the transformation zone, the site of cervical carcinogenesis, is sensitive to estrogenic stimulation. This observation, among others, suggests a link between estrogen and cervical carcinogenesis. Because aromatase is the key enzyme in the final step of estrogen biosynthesis and has direct correlation with estrogen production, we investigated whether aromatase expression is enhanced in cervical carcinomas. All tumors tested (n = 19) were squamous carcinomas, except one that was adenocarcinoma. Our data showed that whereas no aromatase expression was detected in normal cervical tissue (n = 17) or dysplastic samples (n = 42), 35% of cervical carcinomas (n = 19) tested were positive (Fig. 1). Tumors that expressed aromatase were all squamous carcinomas. The data suggest that in situ aromatase expression may be a potential factor involved in the progression of cervical cancer.

Alteration in the expression of steroid receptors and cellular proliferation genes in cervical carcinomas. An increase in the estrogenic activity in normal and cancerous tissue is usually associated with an increase in steroid receptors and cell proliferation–promoting factors. We tested the expression of steroid receptors in normal cervix, precancerous tissue, and cervical carcinomas. Western blot studies show increased protein levels in both ER-α and ER-β, and a decrease in levels of PR mRNA (Fig. 2 A and B). RT-PCR analysis of tissue samples overexpressing aromatase showed a similar pattern of expression at the mRNA levels (Fig. 2C). Interestingly, whereas ER expression showed little or no change between normal and precancerous tissue, PR levels were drastically decreased in precancerous tissue and decreased further in the carcinoma samples (Fig. 2). A previous study has shown that whereas estrogen induces PR expression in the cervix, combination of estrogen and progesterone decreases PR to undetectable levels (29). Similarly, we have shown that the hormonal milieu, whether estrogen by itself or in combination with progesterone, as well as HPV status has varying effects on PR expression in established cervical cancer cell lines (data not shown).

We also examined the expression of cell proliferation factors that are responsive to estrogenic stimulation; namely, cyclin D1, a cell cycle factor, and proliferating cell nuclear antigen (PCNA), a marker of proliferation. Both factors showed a drastic increase in the expression of carcinoma samples as compared with normal cervical tissues (Fig. 2).

Generation of aromatase-overexpressing clones of cervical cancer cell lines. The expression of aromatase in cervical tumor samples and the absence of its detection in any of the normal or precancerous tissue suggests that aromatase induction may lead to increased tumor growth and progression of cervical cancer. To determine what the effect of aromatase, and consequent endogenous estrogen biosynthesis, has on the growth of cervical cancer cells and gene expression, we transfected the aromatase gene driven by the cytomegalovirus promoter into established cervical cancer cell lines. In addition to overexpressing aromatase in HPV+ cells (CaSki, C4I, and HT3), we have also transfected an HPV+ cell line (C33A) with the aromatase construct. Stable transfecants overexpressing aromatase (CaSki-Aro, C4I-Aro, HT3-Aro, and C33A-Aro) were selected for each cell line. To verify the increased expression and activity of aromatase in the transfected lines, we examined aromatase mRNA levels by RT-PCR and the titrated water-release assay, respectively. Representative data are shown for CaSki-Aro and C33A-Aro in Fig. 3. Aromatase expression was increased in C33A-Aro and CaSki-Aro as compared.

![Figure 1. Aromatase expression is induced in cervical carcinomas. Normal cervical tissues, precancerous samples, and cervical carcinomas were examined for aromatase expression by conventional RT-PCR using conditions described in Materials and Methods. Columns, densitometric analysis of aromatase PCR products normalized to the housekeeping gene actin (top). Interrupted distribution of aromatase mRNA expression levels (top). Aromatase expression was confirmed in two representative carcinoma samples (A, C9) using real-time RT-PCR (bottom). Note that no amplification curve was detected for the normal control, suggesting a lack of detectable aromatase mRNA, whereas amplification curves for the carcinoma samples indicate detectable aromatase expression (bottom).](cancerres.aacrjournals.org)
with untransfected controls (Fig. 3A). To confirm that increased aromatase expression corresponds to increased activity, we assayed the activity of aromatase in transfected cell lines using \[^{3}H\]androstenedione as a substrate and, where appropriate, Letrozole as aromatase inhibitor (Fig. 3B and C). As expected, elevated aromatase activity was detected in MCF-7Ca cells, a previously characterized breast cancer cell line that overexpresses aromatase (30). Aromatase activity was enhanced at least 10-fold and 5-fold in CaSki-Aro and C33A-Aro, respectively, as compared with untransfected controls (Fig. 3C).

**Aromatase expression enhances proliferation and colony formation in cervical cancer cells.** To examine whether estrogen has mitogenic effects on cervical cancer cells, we treated several cervical cancer cell lines with exogenous estrogen and examined cellular proliferation in CaSki cells. The dose-response curve in Fig. 4A shows that treatment with 10^-9 mol/L of estrogen is most effective in cellular proliferation. We then examined whether inhibition of estrogen responsiveness by antiestrogen ICI 182,780 results in diminished proliferation in several cervical cancer cell lines (Fig. 4B). Our data show that estrogen does indeed enhance cellular proliferation and ICI 182,780 diminishes this effect (Fig. 4B). We then determined the effects of endogenous estrogen production due to aromatase overexpression on cellular proliferation. A dose-response curve with the aromatase inhibitor Letrozole using CaSki cells showed that a concentration of 1 \( \mu \text{mol/L} \) reduces proliferation by >50% in CaSki-Aro cells (incubated with androstenedione as an aromatase substrate), whereas lower concentrations of Letrozole had a lesser effect on proliferation (Fig. 4C). Adding the aromatase substrate, androstenedione, resulted in increased cellular proliferation of aromatase-overexpressing cell lines (Fig. 4D-G). This induction of proliferation is lowered when Letrozole, an aromatase inhibitor, was added in conjunction with androstenedione, suggesting that local estrogen formed as a result of aromatase activity is responsible for enhanced proliferation (Fig. 4D-G). Interestingly,
androstenedione stimulated proliferation in HT3 and HT3-Aro cells equally, suggesting that HT3 has endogenous aromatase expression (Fig. 4G).

We have examined the effects that exogenous estrogen has on anchorage-independent growth of cervical cancer cells. The data shows that estrogen enhances colony formation in all cells tested; however, the HPV− cell line, C33A, showed the least increase in colony number at <20% (Fig. 5, top). To determine the role of endogenously produced estrogen on cancerous growth, we examined the effects of aromatase on anchorage-independent growth of CaSki-Aro and C33-Aro. Our data show that colony size was larger in CaSki-Aro cells than in CaSki control (Fig. 5A and B). Little difference in colony size was observed between C33A and C33A-Aro cells (Fig. 5C and D). Furthermore, the colony size of CaSki-Aro cells was larger than C33A-Aro cells (Fig. 5E and F).

Cell cycle factors, steroid receptors, and estrogen-responsive growth factors are differentially affected in human papilloma virus−negative and −positive cervical cancer cell lines. To determine whether endogenous estrogen due to aromatase induction has an effect on gene expression related to cellular growth in the presence or absence of HPV infection, we tested the protein levels of cell cycle and proliferation markers, cyclin D1 and PCNA in CaSki-Aro and C33-Aro cells. Western blot analysis in Fig. 6 shows that aromatase overexpression

Figure 4. Estrogen leads to increased cervical cancer cell proliferation. A, estrogen dose-response effects on cervical cancer cell proliferation was measured by MTT assay. B, different cervical cancer cells were treated with estradiol (10−9 mol/L) along with or without ICI 182,780 (10−7 mol/L) and proliferation was measured by MTT assay as described in Materials and Methods. C, Letrozole dose-response effects on CaSki-Aro cellular proliferation was measured by MTT. D-G, aromatase transfected cervical cancer cell lines and corresponding controls were used in proliferation assays. Where indicated, androstenedione (Andr) was added as a substrate for conversion to estrogen by aromatase. Cell proliferation was determined by MTT assay as described in Materials and Methods. Experiments were repeated thrice; *: P < 0.01, representative data.
resulted in little change in the expression of cyclin D1 and PCNA in the HPV− cell line C33A-Aro. However, aromatase overexpression in CaSki-Aro resulted in a 7-fold and 2-fold increase in the expression of cyclin D1 and PCNA, respectively (Fig. 6). Figure 7 shows data demonstrating differential effects of aromatase overexpression in the presence or absence of HPV on steroid receptors and growth factors regulated by estrogen. ER-α and ER-β expression was increased in CaSki-Aro cells as compared with untransfected control (Fig. 7A). No change in ER-α or ER-β expression was detected in C33A-Aro cells (Fig. 7B). Induction of ER in CaSki-Aro is consistent with that of aromatase-overexpressing cervical carcinoma samples, all of which are HPV+. Thus, the induction of estrogen synthesis due to aromatase overexpression may have different effects on steroid receptor expression, depending on HPV infection status.

Transforming growth factor-β (TGF-β), an estrogen-responsive growth factor implicated in a variety of cancers, including cervical cancer, showed increased expression in CaSki-Aro cells and a decrease in C33A-Aro due to aromatase overexpression (Fig. 7B). Expression of EGFR and its ligand TGF-α increased more evidently in C33A-Aro than in CaSki-Aro (Fig. 7B). The estrogen-responsive angiogenic factor vascular endothelial growth factor (VEGF) was increased in both C33A-Aro and CaSki-Aro in response to aromatase overexpression; however, the increase was more pronounced in CaSki cells (Fig. 7B). The data suggests that aromatase induction may contribute to tumor growth by increasing the expression of growth factors and angiogenic factors, such as VEGF, and that the expression pattern of growth factor pathways may depend on HPV status.

**Estrogen receptor activity and expression of human papilloma virus oncogenes E6 and E7 are enhanced in CaSki-Aro cells.** As we have observed that local estrogen synthesis as a result of aromatase expression increases ER expression in CaSki-Aro cells, we examined whether ER/ERE activity increased in these cells as a result of estrogen/ERE-mediated actions. We examined the effects of exogenous estrogen on ER activity in cells transfected with an ERE reporter vector. The data show that estrogen enhances ER activity in both CaSki and C33A cells and that ICI 182,780 diminishes this response (Fig. 8A and B). The data in Fig. 8C shows that aromatase induction in CaSki cells resulted in drastically enhanced ERE-promoter activity, and the addition of Letrozole, an aromatase inhibitor that blocks estrogen synthesis, resulted in diminished promoter induction. Furthermore, cotransfecting with ER-siRNA, an inhibitor of ER-α expression, also resulted in diminished aromatase enhancement of ERE-promoter activity (Fig. 8D). On the other hand, similar studies in C33A-Aro showed lower ERE-promoter activity than CaSki-Aro. This is probably due to the lower expression/activity of aromatase in C33A-Aro cells as compared with CaSki-Aro cells (Fig. 3). Adding exogenous estrogen to C33A-Aro cells resulted in a drastic increase in ERE activity as compared with untreated cells, but was nonetheless lower than that of CaSki cells (Fig. 8). The differences in ERE reporter activity between C33A-Aro cells and CaSki-Aro cells may be due to the lower expression of aromatase in C33A-Aro cells as well as intracellular context differences between the two cell lines, such as ER-α/ER-β ratio, availability of transcriptional cofactors, and HPV status.
HPV is known to have several ERE, rendering E6 and E7 responsive to estrogen stimulation (21). To test whether endogenous estrogen induces these oncogenes, we examined the expression of E6 and E7 in CaSki and CaSki-Aro cells. The data in Fig. 9 show an increase in both E6 and E7 expression, which may contribute to deregulated growth of cancer cells by affecting cell cycle and apoptosis pathways.

Discussion

Estrogen plays a major role in several hormone-dependent malignancies. Aromatase, the enzyme responsible for the rate-limiting and final step of estrogen biosynthesis is mainly expressed in the ovaries and placenta; however, other tissues have been shown to produce aromatase (25). Local aromatase production in breast tissue has been detected and linked with postmenopausal breast cancer (25). Local production of estrogen has also been detected in endometrial cancers and uterine leiomyomas (13, 14, 31–33). Thus, in situ estrogen production may be an etiologic factor that influences the initiation and/or progression of breast and other gynecologic malignancies in the absence of circulating estrogen. The cervix is another tissue responsive to estrogen, and some studies have shown that estrogenic stimulation can influence cervical tumorigenesis (11, 13, 14, 17, 18, 26). In this study, we examined whether in situ estrogen production may be involved in cervical cancer. For this purpose, we compared aromatase expression in cervical carcinoma samples with precancerous tissue and normal cervix. Finding that 35% of cervical cancer samples exhibited detectable aromatase expression along with the absence of detectable aromatase expression in precancerous and normal tissue suggests that aromatase induction may be involved in cervical carcinogenesis and may also influence tumor growth. Based on this novel finding, we have consequently examined the role of estrogen due to induced aromatase levels in cervical carcinogenesis. We detected an increase in the cell cycle factor, cyclin D1, and PCNA, a proliferation marker, both of which are estrogen-responsive and have been implicated in tumor growth. Additionally, we observed an increase in ER-α and ER-β in the tumors, which may be a consequence of increased in situ estrogen synthesis due to aromatase expression.

PR was decreased in dysplastic tissue as compared with normal cervix, and its levels were further decreased in the tumor samples, suggesting that PR loss may correlate with tumor initiation and progression. This is consistent with the observation that disease-free survival was significantly higher in PR+ than in PR− patients who had undergone radiation therapy, underscoring the importance of PR loss in cervical tumorigenesis and its use as a prognostic factor (34). Interestingly, the loss of PR, starting at the precancerous stage, precedes aromatase induction in the tumors. Whether these two events are related is yet to be examined in detail. PR is a known estrogen-responsive gene. Estrogen results in the induction of PR in several tissues of the reproductive tract and the breast, and the mechanisms of PR promoter activation by estrogen have been elucidated in vitro (35, 36). Decreased PR expression in aromatase-expressing tumors suggests that estrogenic control over PR expression may be deregulated in cervical cancer. Interestingly, one study by Gorodeski et al. has shown that treatment of patients with estrogen resulted in increased PR levels in the cervix (29). However, when progesterone was given along with estrogen to patients, PR was drastically lowered to undetectable levels (29). Similarly, we found that estrogen induces...
PR expression in established cervical cancer cell lines; however, the addition of progesterone in combination with estrogen resulted in decreasing PR levels (data not shown). We are currently investigating the role of estrogen and progesterone on the expression of PR in cervical cancer. The data underscores the complex regulation of PR expression in cervical cancer and suggests that a variety of factors may determine the effects of estrogen on PR expression, which may include HPV status, availability of transcriptional cofactors and the stoichiometric ratios of ER-α and ER-β, as well as the local milieu of cytokines, hormones, and growth factors. Very little is currently known about the molecular mechanisms underlying the regulation and actions of PR in cervical cancer cells. Our study points to a complex regulation of PR in cervical cancer that warrants further examination.

It is also of interest to determine whether PR loss and induction of aromatase cooperate in cervical tumor progression. Loss of PR in cervical tumors suggests that it may act as a tumor suppressor in this tissue. A tumor suppressor role for PR has been suggested in ovarian cancer (37). This was based on data from several studies showing that loss of heterozygosity containing the PR locus is common in ovarian cancer cases and that women with progesterone deficiency have an increased incidence of ovarian cancer (37, 38). Progesterone has also been shown to induce apoptosis and regulate apoptosis markers in ovarian cancer cell lines (37, 38). Furthermore, some evidence shows that progesterone may exert its antitumorigenic action by decreasing membrane fluidity, resulting in decreased cell invasiveness and anchorage-independent growth (37). In other studies, it was also shown that progesterone inhibited the invasiveness and secretion of urokinase plasminogen activator in SKOV-3 ovarian cancer cells, which suggests yet another avenue of research in determining the effects of aromatase induction in cervical cancer and PR expression (39).

Increase in levels of steroid receptors and cell proliferation factors in aromatase-expressing cervical tumors suggest that local estrogen biosynthesis, caused by induction of aromatase, may induce the expression of these estrogen-responsive factors, leading to tumor growth. To study this possibility directly, we generated stably-transfected clones of several cervical cancer cell lines, which were then analyzed for PR expression and cell proliferation.

**Figure 8.** Estrogen enhances ER transcriptional activity in CaSki cells. ER/ERE activity was assessed by luciferase reporter assay using consensus ERE-promoter construct. Estrogen (10⁻¹⁰ mol/L) with or without ICI 182,780 was added to cell cultures of CaSki and CaSki-Aro (A) as well as C33A and C33A-Aro (B) transfected with ERE reporter vector. Androstenedione (Andr) was added as a substrate for aromatase, and Letrozole (10⁻⁶ mol/L) was added as an aromatase inhibitor (C). SiRNA specific to ER was used to inhibit ER expression (D). Effectiveness of siRNA-specific suppression of ER-α expression was verified (~90% decrease) by Western blotting, using antibody against ER-α (D, inset). Luciferase assays were done as described in Materials and Methods (*, P < 0.01).

**Figure 9.** Aromatase induces HPV E6 and E7 oncogene expression. RT-PCR was used to determine the effect of aromatase on expression levels of E6 and E7 in CaSki and CaSki-Aro Cells. Primers and conditions used for cDNA amplification are described in Materials and Methods. C, untransfected CaSki control; A, CaSki-Aro cells.
cell lines overexpressing aromatase. The data shows that overexpressing aromatase resulted in increased cellular proliferation and anchorage-independent growth, implicating aromatase in cervical tumor growth.

Using CaSki and C33A cells, we have also examined whether aromatase has different influences in the presence or absence of HPV infection. Consistently, aromatase expression resulted in an increase in both cyclin D1 and PCNA levels in CaSki HPV⁺ cervical cancer cells. However, little change in cyclin D1 and PCNA levels was detected in C33A HPV⁻ cervical cancer cells, suggesting that other alterations in cell cycle genes could possibly cause the aromatase-induced proliferation in these cells. The data also show that aromatase overexpression resulted in increased expression of both ER isotypes as well as ER/ERE activity in HPV⁺ CaSki cells. This suggests that the observed increase in these steroid receptors in aromatase-expressing cervical carcinomas, all of which were HPV⁺, was likely to be a direct consequence of aromatase induction. C33A and C33A-Aro cells showed less ERE activity than CaSki in response to exogenous estrogen treatment or to endogenous estrogen synthesis by aromatase. This suggests that C33A cells may be less sensitive to estrogen than CaSki cells. Nonetheless, the lower response in C33A-Aro cells than CaSki-Aro cells to androstenedione may be due to the lower expression of aromatase in the former cell line and consequent activity resulting in lower conversion of substrate to estrogen. Interestingly, exogenous estrogen treatments to C33A-Aro cells resulted in a >20-fold increase in ERE reporter activity as compared with untreated C33A-Aro cells, yet still significantly lower than the response of CaSki-Aro. This further suggests that CaSki estrogenic response is higher than that of C33A cells, which may be due to differences in cellular factors as well as HPV status.

We observed differences in gene regulation patterns due to aromatase overexpression in CaSki-Aro and C33A-Aro cells. This differential regulation by estrogen may be due to differences in HPV status of the two cervical cancer cells. A possibility exists that the differential expression between the two cell lines may be due to underlying differences other than HPV status, which may require further investigation. Nonetheless, the combined influences of estrogen and HPV infection on cervical carcinogenesis were shown in the K14-HPV transgenic mice in which estrogen treatment resulted in enhanced tumor formation (26). Furthermore, our studies show that aromatase overexpression in CaSki-Aro cells resulted in an increase in the HPV oncogenes, E6 and E7. Thus, it is likely that the interaction between estrogen and HPV has an effect on the observed changes in gene expression, leading to enhanced proliferation of cervical cancer cells.

We also examined the expression of estrogen-responsive growth factors. Aromatase overexpression resulted in decreased TGF-β1 expression and an increase in EGFR and its ligand TGF-α in C33A-Aro cells. Little or no change in the expression of these factors was observed in CaSki-Aro cells. It is possible that increased PR expression in C33A-Aro cells may modulate the repression of TGF-β1, resulting in less aggressive growth than in CaSki-Aro cells, as shown by the anchorage-independent growth data. It would be of interest to determine the regulation of TGF-β2 and TGF-β3 expression in C33A-Aro and CaSki-Aro cells. The data also suggests that aromatase may induce cell growth of C33A cells via an EGFR-dependent pathway. On the other hand, the growth of CaSki cells, in which aromatase induces ER expression and activity may respond to aromatase via the reduction of PR expression and induction of cyclin D1 as well as E6 and E7, two HPV oncoproteins that interfere with cell cycle arrest by inactivating retinoblastoma and p53 proteins, respectively.

In summary, we have detected the induction of aromatase expression in ~ 35% of cervical carcinomas examined. On the other hand, aromatase expression was not detected in any of the normal cervix and precancerous tissues tested. This provides novel evidence implicating in situ aromatase expression in cervical carcinogenesis. We have also shown that aromatase overexpression induces ER levels and activity in HPV⁺ cervical cancer cells, and this increase is associated with induction of the expression of the viral oncoproteins, E6 and E7. This study suggests that aromatase induction may be a causative agent in cervical carcinogenesis and tumor growth, and it raises the possibility that aromatase inhibitors may be used as a potential line of therapy for aromatase-positive cervical tumors. Furthermore, ongoing experiments using immunohistochemistry in a large number of cervical carcinoma samples will help determine the prognostic significance of aromatase expression.

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**References**

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