Increased Serum Progesterone and Estradiol Correlate to Increased COX-2 Tissue Expression in Cervical Intraepithelial Neoplasia

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Abstract. The aim of this study was to investigate correlations between serum progesterone and serum estradiol levels and expression of tissue tumor markers in cervical intraepithelial neoplasia (CIN) and normal epithelium. Materials and Methods: Eighty women of fertile ages with cervical biopsies ranging histologically from normal to CIN III were included. Expression of eleven tumor markers was studied. Serum levels of progesterone and estradiol were analyzed. Exclusion criterion was hormonal contraceptive use. Results: In normal epithelium, low progesterone levels correlated to expression of epidermal growth factor receptor (EGFR) and CD4+. In initial analyses of CIN, high progesterone levels correlated with expression of retinoblastoma protein, p16 and cyclooxygenase-2 (COX-2), but after adjustment for CIN grade, only correlation to COX-2 expression remained significant. Expression of COX-2 and CD4+ correlated to serum estradiol levels in CIN. Conclusion: Serum levels of progesterone and estradiol appear to correlate with increased COX-2 expression in CIN. In addition, the study shows that evaluation of expression of tumor markers must take into account the grade of CIN.

A number of tumor markers have been studied in cervical intraepithelial neoplasia (CIN). The objectives have generally been to find tumor marker expression that could discriminate between normal cervical epithelium and CIN, or aid the histological diagnosis of CIN grade. Other studies have focused on determination of the aggressiveness of CIN, but with no conclusive results. During recent years, much attention and extensive research has been directed towards studies of p16, retinoblastoma protein (Rb), p53 and Ki-67/MIB1 (1-3).

The epidemiological correlation between long-term oral contraceptive (OC) use and cervical neoplasias is well established, and OC use has been accepted as a risk factor since the mid 1980s, when a number of studies were able to control for sexual risk behavior (4, 5). It seems plausible that there is a biological role for sex steroid hormones in the development of cervical neoplasms, but the underlying mechanisms are virtually unknown and available results are, in general, restricted to in vitro studies on cervical cancer cell lines (6-12).

In previous studies on invasive cervical squamous cell cancer we showed that serum estradiol and serum progesterone levels correlated with the DNA growth fraction in fertile women (13). Women with a fatal outcome had a longer survival when serum estradiol levels were high and serum progesterone levels were low (14).

The aim of the present study was to evaluate eleven tumor markers and their correlation, if any, to serum progesterone and serum estradiol levels. The women included in the study were premenopausal with regular menstruations. OC users and those with gestagen-only contraceptives were excluded. Some tumor markers based on our previous results on invasive cervical cancer (15), and those that were previously studied in cervical neoplasias which could be of possible clinical importance were analyzed. Tumor markers were chosen to represent different mechanisms in carcinogenesis.

Materials and Methods

The study population comprised 80 women. Sixty women were consecutively recruited from the Out-patient Surgery, Department of Obstetrics and Gynecology, Falun Hospital, and were to undergo a laser conization of the cervix uteri because of CIN or suspicious CIN; atypical squamous cells of unknown significance (ASCUS). Inclusion criteria were premenopausal status, regular menstruation, and no oral contraceptive or gestagen-only contraceptive use. Menstrual cycle phase did not influence the recruitment. After
In brief, glass slides were deparaffinized in xylene (2×15 min), invalid diagnoses. blocks, containing 24 to 30 punch biopsies on average. Each TMA microscopically reviewed by one of the authors (TT) and the most standard methods. Sections from the original paraffin blocks were contraceptive use and smoking habits were given to the participants. Serum progesterone and serum estradiol were analyzed by standard methods. Sections from the original paraffin blocks were microscopically reviewed by one of the authors (TT) and the most representative area(s) was marked for tissue microarray (TMA). One two-millimeter punch biopsy was taken from the blocks corresponding to the marked area and joined into TMA paraffin blocks, containing 24 to 30 punch biopsies on average. Each TMA block also included one control and one empty square to avoid invalid diagnoses.

Immunohistochemistry was performed as previously described (16). In brief, glass slides were deparaffinized in xylene (2×15 min), dehydrated through graded alcohols and endogenous peroxidase was blocked (using H₂O₂ in 70% ethanol). Antigen retrieval was performed using Target Retrieval Solution (TRS pH 6.0 or pH 9.0; Labvision, CA, USA) in a decloaking chamber (Biocare Medical, Walnut Creek, CA) for 4 minutes at 125°C. Thereafter the slides were immunostained in an automated staining instrument, where slides were incubated with primary antibodies and secondary reagent (see Table I) 30 minutes at room temperature (RT). Finally, the slides were incubated with diaminobenzidine (DAB) as chromogen for 10 min and counterstained with Mayer's hematoxylin (Sigma-Aldrich, St Louis, MO, USA) for 15 min. Slides were washed in distilled water for 10 min, dehydrated through graded alcohols to xylene, and mounted in Pertex organic mounting medium (Histolab, Gothenburg, Sweden).

Details of the 11 antibodies chosen for the study are given in Table I. One of the authors (GP) who was blinded for clinical details evaluated the tumor marker expressions and consensus was achieved with another pathologist (TT) in uncertain cases. All the reviewed cases were categorized as normal, CIN I, CIN II, CIN III, or as borderline (if showing some but not all of the following features: mitotic figures, vertical nuclear growth pattern, perinuclear halo, indistinct cytoplasmic border and no primitive cells in upper 1/3 of squamous layer). Frequency of immunostained cells and intensity of staining were evaluated. A four-grade semi-quantitative score was used for frequency, where 0 represented absence of biomarker expression and 1-3 represented increasing percentages of immunopositive cells (1-19%, 20-49% and ≥50%, respectively). Intensity of staining was graded in four steps: absent, weak, moderate and strong. In the analyses there was, in general, a good correlation between frequency and intensity and the best discriminatory evaluation was used for presentation in Tables.

For the analyses, the most explanatory cut-off level was used when the results were dichotomized. When there was no evidence of any correlation to serum progesterone and/or estradiol, data were dichotomized so that an equal number of patients, if possible, were included in the two groups. For adjustments according to CIN grade, the material was divided into high-grade (CIN II and CIN III) and low-grade (borderline and CIN I) squamous intraepithelial lesions (HSIL and LSIL, respectively) and logistic regression was used. For statistical analyses, the JMP statistical package (SAS Institute) was used. Student’s t-test was used for significance testing. The study was approved by the Research Ethical Committee, Uppsala University.

**Results**

The mean and median age was 35.7 years and 34.5 years, respectively, with a range of 22-52 years. Twenty-four (30.0%) women were smokers. The diagnoses were distributed as 25 (31.5%) cases of normal epithelium, 10 (12.5%) borderline, 18 (22.5%) CIN I, and 27 (33.8%) CIN II-III.

Table II gives the results of serum progesterone levels and their correlations to tumor marker expression. Positive or negative correlations between serum progesterone levels and intensity of expression were evident in CIN for Rb, p16, and cyclooygenase-2 (COX-2) in crude analysis. After adjustment for CIN grade, only COX-2 expression remained significantly correlated with serum progesterone levels. In normal epithelium, epidermal growth factor receptor (EGFR) and CD4⁺ expression correlated with low serum progesterone levels. In HSIL there was a higher correlation between COX-2 expression and high progesterone levels (17.5 vs. 6.6 nmol/l; p=0.009).

**Discussion**

It is evident that serum estradiol and progesterone levels show associations with tumor markers that are involved in proliferation, immunological mechanisms and multiple functions (COX-2) in CIN and normal epithelium. The strongest correlation was observed between high serum progesterone levels and COX-2 expression in high grade CIN.

This is the first study of its kind and correlations between serum sex steroid hormones levels might help us to understand the mechanisms behind epidemiological findings, such as long-term OC use and parity. Both progesterone and, to some extent, estrogens have been claimed to induce cervical cancer in *in vitro* studies.

In cancer studies, the proteins that were included here are generally referred to as tumor markers. We chose to present the results of expression in normal epithelium and those with LSIL/HSIL. An interesting finding was significant correlations that vanished when adjustment was made for the
grade of CIN. Differential expression of tumor markers in LSIL and HSIL explain these results and must be kept in mind in any similar study.

Intense staining of EGFR correlated with low progesterone levels in normal epithelium, but not in CIN. Progesterone is a suppressor of endometrial growth, but the effect on squamous cervical epithelium has been poorly studied. A previous study did not show any correlation to CIN grade or human papillomavirus (HPV) infection (17). An increased staining intensity of EGFR was reported with CIN progression, mainly because expression extended from the basal to superficial epithelial layers (18).

The retinoblastoma pathway is closely associated with p16INK4a expression. The HPV E7 gene product inactivates
Rb by phosphorylation, resulting in a negative feedback loop with increased p16\(^{INK4a}\) expression, but as the Rb response is poor it will lead to a continuously high p16\(^{INK4a}\) expression. p16\(^{INK4a}\) serves as a surrogate marker for HPV infection, but its clinical significance is obscure (1-3, 19). Our finding of a diminished correlation between strong Rb and low p16 expression and very high progesterone levels when adjustment for CIN grade was made seems likely to reflect the higher frequency of HPV infection in HSIL. The inverse relationship between high p16\(^{INK4a}\) expression and low Rb expression confirms the correlation between these tumor markers in HPV infections.

Expression of another two tumor suppressor proteins were included in the study, fragile histidine triad (FHIT) and p53. The FHIT gene is located at chromosome 3p and is frequently lost in many cancer types. The FHIT gene product acts as a tumor suppressor, one mechanism is via its proapoptotic activity. A progressive loss is seen in cervical neoplasias and invasive cancer (20, 21). In the present study, no correlation between high serum progesterone or estradiol and altered FHIT expression was found.

The last tumor suppressor included, p53, did not correlate to progesterone levels. p53, like Rb, was one of the first detected tumor suppressors and one of the major mechanisms of p53 action is cell-cycle arrest, allowing for DNA repair or apoptosis of damaged cells. In most cancer types, mutant p53 is found. Interestingly, in cervical cancer the wild-type p53 dominates. p53 is, however, degraded by the HPV E6 gene product, in analogy with the Rb protein (22). In a previous study of invasive cancer, we found significantly lower p53 expression with high serum progesterone levels (15).

COX-2 responds to a variety of mitogenic and inflammatory stimuli and is thought to be involved in a number of steps in cancer development, in itself or through prostaglandins. Among these are apoptosis, proliferation, immune suppression and neoangiogenesis. Evaluations of a clinical role for COX-2 expression indicate an association with poor prognosis in cervical cancer (23, 24). The finding in this study, a correlation between strong intensity of COX-2 expression and elevated serum progesterone levels, in particular in HSIL, suggests an adverse effect of progesterone on CIN. Similar correlations were found for serum estradiol levels.

Cytokines are released in response to infection of the uterine cervix by HPV among others. Interleukin-10 (IL-10) is a T-helper cytokine and has a suppressive effect on cell mediated immunity. Increased expression has been found to be associated with progressive cervical neoplasia and cancer (25, 26). In the cervix IL-10 facilitated establishment of HPV infections by inhibiting immune responses (26). In the present study, high progesterone levels did not correlate to IL-10 expression.

### Table III. Serum estradiol and correlation to tumor marker expression in CIN and normal squamous cell epithelium.

<table>
<thead>
<tr>
<th>Tumor Marker</th>
<th>Frequency</th>
<th>Normal Epithelium</th>
<th>CIN</th>
<th>p-Value</th>
<th>Normal Epithelium</th>
<th>CIN</th>
<th>p-Value</th>
<th>p-Value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclooxygenase-2 (COX-2): frequency 2-3 vs. 0-1</td>
<td>31</td>
<td>587.8 vs. 420.0</td>
<td>0.22</td>
<td>45</td>
<td>518.7 vs. 340.3</td>
<td>0.04</td>
<td>0.046</td>
<td></td>
</tr>
<tr>
<td>Epidermal growth factor receptor: frequency 1-3 vs. 0</td>
<td>30</td>
<td>420.1 vs. 785.8</td>
<td>0.06</td>
<td>39</td>
<td>399.1 vs. 569.5</td>
<td>0.40</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Retinoblastoma protein: frequency moderate/strong vs. none/light intensity p16: light/strong vs. none intensity</td>
<td>29</td>
<td>511.3 vs. 327.0</td>
<td>0.28</td>
<td>36</td>
<td>451.1 vs. 338.4</td>
<td>0.32</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>Fragile histidine triad (FHIT): frequency 2-3 vs. 0-1</td>
<td>29</td>
<td>430.8 vs. 474.3</td>
<td>0.80</td>
<td>40</td>
<td>391.1 vs. 500.8</td>
<td>0.45</td>
<td>0.68</td>
<td></td>
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<tr>
<td>CD4*: frequency moderate/strong vs. none/light intensity</td>
<td>31</td>
<td>470.0 vs. 454.4</td>
<td>0.93</td>
<td>45</td>
<td>394.5 vs. 423.7</td>
<td>0.89</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Interleukin 10 (IL10): frequency 1-3 vs. 0</td>
<td>29</td>
<td>512.1 vs. 446.7</td>
<td>0.70</td>
<td>40</td>
<td>348.3 vs. 464.8</td>
<td>0.18</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Cytokeratin 10 (CK10): frequency moderate/strong vs. none/light intensity p53: frequency 3 vs. 0-2</td>
<td>30</td>
<td>508.1 vs. 453.9</td>
<td>0.73</td>
<td>40</td>
<td>400.6 vs. 424.9</td>
<td>0.83</td>
<td>0.80</td>
<td></td>
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<tr>
<td>KI-67: strong vs. none/moderate intensity</td>
<td>29</td>
<td>493.0 vs. 455.3</td>
<td>0.89</td>
<td>38</td>
<td>638.0 vs. 372.9</td>
<td>0.11</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>E-cadherin: strong vs. none/moderate intensity</td>
<td>29</td>
<td>493.4 vs. 429.5</td>
<td>0.75</td>
<td>38</td>
<td>381.5 vs. 554.1</td>
<td>0.12</td>
<td>0.14</td>
<td></td>
</tr>
</tbody>
</table>
| Hormonal contraceptive users and postmenopausal women were excluded. <sup>a</sup>Adjusted for HSIL vs. LSIL. Intensity was measured as none, light, moderate and strong staining; frequency of staining was measured semiquantitatively: 0: 0%, 1: 1-19%, 2: 20-49% and 3: ≥50% stained cells.
Cellular immune responses, most important by antigen-specific T lymphocytes, are considered the critical defence mechanisms against HPV-infected cells. CD8+ responses are involved in the generation and clonal expansion of cytotoxic T lymphocytes. One role for CD4+ T-cells is to help during the generation of CD8+ T-cell responses (27). The correlation between absence of CD4+ expression and high progesterone levels in normal epithelium could not be analyzed in CIN because of almost universal expression of CD4+.

Progesterone has previously been suggested as a major candidate risk hormone in cervical neoplasias, although the present study is the first to investigate molecular biological mechanisms. Estrogen has been reported to reduce susceptibility to primary HPV infection, but might be of no importance once an HPV infection has been established (28). HPV has a tendency to transfect cells with progesterone receptors. Both HPV 16 and HPV 18 contain progesterone and glucocorticoid response elements that increase expression of the HPV E6 and E7 oncogenes, considered crucial in cell transformation (6). Such a transformation has been reported to take place when progesterone or OC gestagens were added to cell cultures (8).

In an experimental study (9), an enhanced colony-forming efficiency was found in the HPV 16-DNA-integrated cervical cancer cell line, CaSki, after at least three days of progesterone treatment. The progesterone antagonist RU 486 was able to abrogate the enhancement of progesterone on cell growth. Progesterone and glucocorticoid hormones increase HPV mRNA and significantly stimulate viral replication (29). Progesterone response elements occur in the long control regions of HPV 16 in the cervical cancer cell line SiHa and are thought to mediate an increase in transcripts encoding E6 and E7 proteins (6). Estrogen-treated SiHa cells were also found to induce HPV transcripts, while there was no such effect when progesterone was added (7). An increased cell proliferation with high serum progesterone levels in invasive squamous cell cervical cancer has also been reported in a study which included more than 100 women (14).

When transgenic mice expressing HPV 16 were treated with estrogens, squamous cell carcinomas developed exclusively in the transformation zone (30). Estrogens might also have a role in increasing the levels of HPV-induced apoptosis (10) or attenuating p2X7-R-mediated apoptosis (12). Experimental studies have produced conflicting results, particularly in studies of estrogens. Experimental studies suffer from the problem of introducing correct, physiological concentrations of hormones and might be a cause for discrepant results in different studies. Sex steroid hormones might also be involved in single steps in the neoplastic progression, similarly to that of many tumor markers. Once invasive cancer has been established, high serum estrogen levels might have a positive effect on outcome, while high serum progesterone levels have been found to have a deleterious effect (14).

Some previous studies have studied sex steroid hormone interactions with HPV, in vitro experiments on cervical cancer cell lines, and animal experiments. This clinical study on women with normal or neoplastic epithelium indicates that serum progesterone and estradiol levels influence cellular and extracellular proteins which have been associated with neoplastic development, in normal epithelium and CIN. Future studies should involve additional biological markers of possible importance in CIN and invasive cervical cancer.

References


