Regression of Latent Endometrial Precancers by Progestin Infiltrated Intrauterine Device

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Abstract
PTEN tumor suppressor inactivation is the earliest step in endometrial carcinogenesis, occurring in morphologically unremarkable endometrial glands in half of normal women. We test the hypothesis that sex hormones positively or negatively select for these "latent precancers" by examining their emergence, persistence, and regression rates under differing hormonal conditions. Perimenopausal and postmenopausal women had an intake endometrial biopsy and underwent hormonal therapy with progestin-impregnated intrauterine device (IUD; n = 21), cyclic oral progestins (n = 28), or surveillance only (n = 22) with follow-up biopsies. For comparison, premenopausal naturally cycling endometrial biopsies were studied as single time points in 87 patients and multiple surveillance time points in 34 patients. Biopsies in which any PTEN protein-null glands were found by immunohistochemistry were scored as containing a latent endometrial precancer. All groups had a similar proportion of latent precancers at intake but differed after therapy. Emergence rates were highest (21%) for the naturally cycling premenopausal group compared with just 9% for untreated perimenopausal women. The IUD group had the highest rate of regression, with a 62% pretherapy and 5% post-therapy rate of latent precancers. This contrasted to nonsignificant changes for the oral progestin and untreated control groups. Delivery of high doses of progestins locally to the endometrium by IUD leads to ablation of preexisting PTEN-inactivated endometrial latent precancers and is a possible mechanism for reduction of long-term endometrial cancer risk known to occur in response to this hormone. (Cancer Res 2006; 66(11): 5613-7)

Introduction
Endometrioid (type I) endometrial adenocarcinoma, the most common form of endometrial cancer, is a hormonally responsive tumor that has been associated with the exposure risk factor of estrogens unopposed by progestins and, in up to 83% of cases, inactivation of the PTEN tumor suppressor gene (1). Unopposed estrogens increase the risk for this malignancy 3- to 10-fold (2). Although estrogens increase the risk for this malignancy, they develop any histologic phenotype or even clinically measurable increased cancer risk (8). Hormonal selection of preexisting mutated clones is one possible mechanism of risk stratification, which explains known hormonal epidemiologic and molecular genetic data in a unified model.

The PTEN tumor suppressor gene is a useful biomarker for the earliest stages of carcinogenesis, as it is mutated in up to 83% of sporadic endometrioid endometrial cancers (9), induces endometrial cancer on inactivation in mice (10), and is inactivated well in advance of development of established disease. Normal premenopausal naturally cycling women (43%) have small numbers of histologically diagnosable premalignant lesions suggest that they develop any histologic phenotype or even clinically measurable increased cancer risk (8). Hormonal selection of preexisting mutated clones is one possible mechanism of risk stratification, which explains known hormonal epidemiologic and molecular genetic data in a unified model.

There is evidence that latent precancers specifically defective in the PTEN gene are linked to hormonal modification of endometrial cancer risk. PTEN-defective latent endometrial precancers maintain high levels of nuclear estrogen and progesterone receptors (8). Physiologic expression of endometrial gland PTEN protein is greatest in a mitotic, estrogen-rich environment when its tumor suppressor functions are required to control the rate of cell division (12). Under estrogen stimulation, PTEN-mutant cells would thus be...
expected to have a selective proliferative advantage, which is lost on progestin exposure when even genetically intact glands shut down PTEN expression. Under conditions of a normal monthly menstrual cycle, the progestin exposures are insufficient to ablate latent precancers, only 17% of which disappear a year later (8). If the dose and duration of progestins are increased to therapeutic levels, PTEN-mutant latent precancers undergo a 90% rate of involution, thereby resetting the carcinogenesis “clock” (13).

The current study further tests the hypothesis that ablation of preexisting PTEN-defective endometrial latent precancers is a potential mechanism for progestin reduction of endometrial cancer risk. From our previous work, we know that immunohistochemically PTEN-null glands are likely to harbor PTEN mutations and/or deletions (8, 9). Therefore, we used highly sensitive PTEN immunohistochemistry to detect changes in the prevalence of latent precancers that occur over time in the endometria of women undergoing defined hormonal therapies. Latent precancer rates were determined before and after therapy for women undergoing low-dose intermittent oral progestin therapy and high-dose local progestin delivery by an intrauterine device (IUD). Results are compared with a comparable age group undergoing routine gynecologic care in the perimenopausal period and data from premenopausal naturally cycling women.

Materials and Methods

Patient selection. Women in northern Norway (Tromso region) with successive endometrial biopsies taken under different hormonal conditions form the three main experimental groups of this study. Clinical aspects of these patients have been published elsewhere (14, 15) but will be summarized here. Patients presenting with symptomatic endometrial bleeding and a diagnosis of endometrial hyperplasia had an intake endometrial biopsy followed by treatment either with a progestin-impregnated IUD [group 1, IUD releasing 20 μg levonorgestrel daily (Levonova device, Schering, Turku, Finland)] or systemic oral progestin (group 2, 10 mg medroxyprogesterone) administered daily for 10 days a month and repeated for 3 months. Patients were rebiopsied after receiving therapy. A comparison group [group 3] of women with successive biopsies undergoing clinical surveillance for management of perimenopausal symptoms and signs constituted a progestin “untreated” group from the same patient population. Just under half (10 of 22 reported in Results) received low-dose hormonal replacement therapy as follows: 1 mg norethisterone/2 mg estradiol daily (6 patients), 1 to 2 mg estroral daily (3 patients), 2.5 mg tibolone daily (1 patient), and no therapy (12 patients). All patient materials were compiled from existing pathologic tissues (3 patients), 2.5 mg tibolone daily (1 patient), and no therapy (12 patients).

Pathology materials. Pretreatment and post-treatment archival paraffin-embedded blocks containing endometrial biopsy tissue were available for 26 IUD-treated, 30 oral progestin-treated, and 28 untreated control patients and single blocks from 99 proliferative reference patients. All post-treatment biopsies were obtained while still on hormonal therapy. Tissue amount was deemed adequate if a minimum of five endometrial glands or equivalent quantities of dislodged endometrial epithelium were identified. Of these, 22 were rejected because of inadequate amounts of endometrial tissue to do immunohistochemistry. 1 control was rejected because of active progestin implant (Implanon) treatment, and 8 were rejected because of high background or other artifact during PTEN immunohistochemistry itself. This left paired biopsies from 21 IUD-treatment, 28 oral progestin-treated, and 22 untreated control patients and single blocks from 87 proliferative reference patients for whom complete diagnostic, sampling interval, and immunohistochemical results are available and reported in Results.

Pathologist diagnostic review using endometrial intraepithelial neoplasia criteria. Slides were diagnosed according to endometrial intraepithelial neoplasia (EIN) terminology by a gynecologic pathologist (G.L.M.) using published criteria (16, 17). Areas diagnosed as EIN were required to meet four criteria: (a) area of glands exceeds area of stroma; (b) when a localizing lesion is present, epithelial cells within the architecturally crowded focus were cytologically different compared with background; (c) area meeting architectural and cytologic criteria must have a minimum size of 1 mm; and (d) exclusion of mimics and carcinoma. Endometrioid polypos are localizing lesions that met at least two of the following three diagnostic criteria in an area confirmed to be endometrial functionalis: (a) irregular gland architecture, (b) altered stroma, and (c) thick-walled vessels.

PTEN immunohistochemistry. Paraffin sections of endometrial biopsy and curettage specimens were rehydrated and underwent antigen retrieval by microwave before overnight (4°C) incubation with 1:300 murine monoclonal anti-PTEN antibody 6H2.1 (Cascade BioScience, Winchester, MA) as described previously (8). Slides were washed, incubated with appropriate secondary biotinted immunoglobulin (Vectastain avidin-biotin complex method kit, Vector Laboratories, Inc., Burlingame, CA), and signal detected by sequential addition of avidin peroxidase and 3,3′-diaminobenzidine. Slides were counterstained with methyl green and coverslipped.

Each block underwent PTEN staining in independent duplicate immunohistochemical runs that included standard known slides as a run quality control. Staining adequacy was assessed by internal positive control staining in the slide of interest (normal endometrial stroma), a negative control of each slide in which primary antibody had been omitted, and review of run controls. PTEN status was scored visually (by G.L.M.) as “PTEN null” if any endometrial glands devoid of PTEN protein were seen and “PTEN normal” if all endometrial glands visualized expressed PTEN protein. Null glands were generally devoid of PTEN protein in all component cells, recognizable by reference to immediately adjacent PTEN-staining stroma.

Data reduction. Data were entered into an excel spreadsheet, which was imported into Systat version 11 (SYSTAT Software, Inc., Point Richmond, CA) for statistical analysis.

Results

Clinical and demographic characteristics of all patient groups are shown in Table 1. Histologic diagnosis of intake biopsies is shown in Table 2.

Patient age was similar within the separate perimenopausal (groups 1-3, average 48-53 years) and premenopausal (groups 4-5, average 41-42 years) groups but, as expected, differ between them.
The prevalence of latent precancers at initial presentation (no treatment) was similar between all five groups studied ($\chi^2; P = 0.077$). The proportion of latent precancers in follow-up biopsies, however, showed significant differences between the four available groups ($\chi^2; P = 0.002$), suggesting a possible effect of intervention.

Sequential sampling before and after an interval of treatment provided an initial reference point (pretreatment) to determine the overall magnitude and significance of a post-treatment effect. This was analyzed by pretherapy and post-therapy group comparison of latent precursor proportions (Table 3). “Post-therapy” refers to samples obtained following a specified duration of hormonal treatment while therapy was still being administered. The only group that showed a significant change overall in the proportion of PTEN-null endometria over time is the IUD-treated patients (Fisher’s exact test; $P < 0.001$). Pretreatment endometria (62%) contained PTEN-null glands, declining to only 5% after an average of 49 days of treatment with the progestin-impregnated IUD.

The pattern of change in latent precancers in paired samples over time in individual patients provides some insights into the balance of PTEN-null gland emergence, persistence, and regression events within each treatment group (Table 4). Patients in each group (29-50%) had no PTEN-null clones at any time during the study. Those patients who had a minimum of one biopsy with a latent precursor were classified as (a) emergent, when only the second biopsy contained a latent precursor, (b) persistent, when both the first and second sample have a latent precursor (Fig. 1A), and (c) regressed, when a latent precursor seen in the first biopsy was absent in the second (Fig. 1B). Highest regression rates were seen in the IUD group, where 62% of all patients had a latent precursor at intake and all of these regressed by the second biopsy ($\chi^2; P < 0.001$). Highest emergence rates were seen in the (untreated) groups of normal endogenously cycling proliferative endometria (group 5, PE2), where 21% of all patients developed a new latent precursor during follow-up, but this was not statistically significant ($\chi^2; P = 0.116$). Persistence rates were highest in the endogenously cycling proliferative endometria (29%) and in the cyclic low-dose oral progestin perimenopausal (35.7) group ($\chi^2; P = 0.006$).

### Discussion

Changes in endometrial latent precursor rates, as detected by PTEN immunohistochemistry, were studied in untreated controls and in women either taking cyclic low-dose oral progestins or having placement of a progestin-impregnated IUD. All groups initially had comparable proportions of patients whose endometria contained PTEN-null glands, but these diverged significantly after therapy. The progestin-impregnated IUD group, which delivered the highest local dose of progestins for the longest period, experienced a strong trend toward regression of preexisting latent precancers, with all latent precancers seen at intake disappearing on post-therapy follow-up. Involution rates were modest and statistically insignificant for the other treatment and control groups.

Progestins are capable of inducing dose-dependent apoptotic cell death of neoplastic endometrial cells grown in culture but with a rapid extinction over a period of a few days. Subsequent withdrawal is accompanied by resumption of apoptotic cell death on a scale several orders of magnitude greater than achieved in the preceding steady state (18). Dose and schedule of administration therefore interact in defining the net effect. Local delivery of progestins by placement of an impregnated IUD provides a very high endometrial concentration of hormone while diminishing the complications of systemic distribution. Such devices have even been effective in treatment of established well-differentiated endometrial adenocarcinoma (19) and present nonsurgical alternative therapies for management of premalignant endometrial lesions.

Declines in preexisting latent precancers were not seen in endogenously driven normal menstrual cycles and in cyclic oral administration of low-dose progestins. It is entirely possible that the cancer-protective effects seen in low-dose oral progestin and
from an intact gene. In our hands, loss of PTEN protein as assessed gene itself rather than transient alteration of protein expression these are due to irreversible changes in the structure of the detection of PTEN protein-null endometrial glands and know that control of cell division rates confer proliferative and survival functions, including Akt-dependent enabling of apoptosis, and nant phases of disease (9). Loss of PTEN tumor suppressor establishes lineage continuity between premalignant and malig-

Table 3. Prevalence of PTEN-null endometria by groups

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Group name</th>
<th>n</th>
<th>Pretreatment, % (n)</th>
<th>Post-treatment, % (n)</th>
<th>Interval (days), mean (median)/range</th>
<th>( P ) (Fisher’s exact test) unpaired</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IUD</td>
<td>21</td>
<td>62 (13)</td>
<td>5 (1)</td>
<td>251 (161)/38-1,142</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>P-cycle</td>
<td>28</td>
<td>68 (19)</td>
<td>39 (11)</td>
<td>332 (154)/38-1,248</td>
<td>0.060</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>22</td>
<td>41 (9)</td>
<td>18 (4)</td>
<td>442 (252)/43-1,601</td>
<td>0.185</td>
</tr>
<tr>
<td>4</td>
<td>PE1</td>
<td>87</td>
<td>49 (43)</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>PE2</td>
<td>34</td>
<td>35 (12)</td>
<td>50 (17)</td>
<td>401 (400)/26-1,167</td>
<td>0.327</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>105</td>
<td>50 (96/192)</td>
<td>31 (33/105)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( \chi^2 \) \( P = 0.077 \) \( P = 0.002 \)

Table 4. Latent precancer absence, emergence, persistence, and regression in successive samples of individual patients

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Group name</th>
<th>n</th>
<th>Absence, % (n)</th>
<th>Emergence, % (n)</th>
<th>Persistence, % (n)</th>
<th>Regression, % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IUD</td>
<td>21</td>
<td>33.3 (7)</td>
<td>4.8 (1)</td>
<td>0 (0)</td>
<td>61.9 (13)</td>
</tr>
<tr>
<td>2</td>
<td>P-cycle</td>
<td>28</td>
<td>28.6 (8)</td>
<td>3.6 (1)</td>
<td>35.7 (10)</td>
<td>32.1 (9)</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>22</td>
<td>50 (11)</td>
<td>9.1 (2)</td>
<td>9.1 (2)</td>
<td>31.8 (7)</td>
</tr>
<tr>
<td>5</td>
<td>PE2</td>
<td>34</td>
<td>44.1 (15)</td>
<td>20.6 (7)</td>
<td>29.4 (10)</td>
<td>5.9 (2)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>105</td>
<td>39 (41)</td>
<td>10.5 (11)</td>
<td>21 (22)</td>
<td>29.5 (31)</td>
</tr>
</tbody>
</table>

\( \chi^2 \) \( P = 0.383 \) \( P = 0.116 \) \( P = 0.006 \) \( <0.001 \)

NOTE: Each patient was assigned to one of four groups depending on the presence or absence of immunohistochemically detected PTEN-null glands (latent precancers) in successive (first and second as shown in Fig. 1) biopsies over time: absence (first and second biopsies without PTEN-null glands), emergence (PTEN-null glands in second but not first biopsy), persistence (PTEN-null glands in both first and second biopsy), and regression (PTEN-null glands seen in first biopsy are not seen in second). For each group (row), the percentage of patients with latent precancers having a pattern of absence, emergence, persistence, or regression is shown.

Combination contraceptive administration require a longer dura-
tion to achieve than the relatively short-term follow-up in this study. There was a measurable but not statistically significant difference, however, in the emergence of new latent precancers during follow-up between these groups, with 21% of all cycling proliferative patients developing emergent latent precancers during follow-up, compared with only 4% to 9% in the more quiescent endometria of the three perimenopausal groups (groups 1, 2, and 3). This is no surprise, as random mutagenesis, the presumed mechanism of origin for most latent precancers, is expected to occur in proportion to the mitotic activity of the source tissue (7, 20).

Despite a high rate of acquired PTEN mutation in histologically normal tissues and a correspondingly low lifetime incidence of endometrial cancer, there are good reasons to link inactivation of PTEN to endometrial carcinogenesis. PTEN knockout mice develop endometrial carcinoma in 20% of cases (10). PTEN mutation is the most common genetic defect in endometrioid endometrial adenocarcinomas (21). Carry forward of exact PTEN mutations seen in precancers to subsequent cancers in the same patient establishes lineage continuity between premalignant and malignant phases of disease (9). Loss of PTEN tumor suppressor functions, including Akt-dependent enabling of apoptosis, and control of cell division rates confer proliferative and survival advantages long associated with the neoplastic phenotype (1).

We have previously used immunohistochemistry as a tool for detection of PTEN protein-null endometrial glands and know that these are due to irreversible changes in the structure of the PTEN gene itself rather than transient alteration of protein expression from an intact gene. In our hands, loss of PTEN protein as assessed by immunohistochemistry is highly associated with presence of mutations of the PTEN gene or deletions of the 10q23 locus for PTEN (8, 9, 22, 23). For small numbers of paraffin-embedded endometrial glands isolated by laser capture microdissection, we have found that all PTEN-expressing glands identified by immunohistochemistry have a wild-type (normal) genotype, whereas 84% of nonexpressing samples have either a mutation or a loss of at least one 10q23 heterozygous marker in the region of the PTEN locus (8). A key aspect of achieving such a high concordance between genetic inactivating events and loss of protein by immunohistochemistry is use of appropriate antibodies (not all commercially available reagents meet this standard; ref. 24).

Sampling error must be considered as a variable in monitoring of latent precancers for purposes of defining their fate in response to interventional therapies. Typically, only a very small number of affected glands are present in a single biopsy, representing <2% to 3% of all present. Although this is an advantage in providing abundant positively staining glands within the same specimen for interpretive comparison of loss of signal, they may be missed in a single specimen due to simple sampling error. This must account for some fraction of "emergent" precancers that were seen after therapy but missed at intake. For this reason, we have described these events as relative comparisons between therapeutic subgroups of patients that share the same sampling errors.

A potential problem in studying physically small premalignant lesions is that the biopsy itself may be destructive, removing the affected cells exclusive of a treatment effect. Data of persistence over time in normal proliferative endometrium suggest that sufficient PTEN-null glands usually remain after biopsy to be detected in
Hormonal Ablation of Latent Endometrial Precancers

Figure 1. Fate of latent endometrial precancers under differing hormonal conditions. A, persistence of PTEN-null glands following 491 days of treatment with a progestin-impregnated IUD. Secretory exhausted glands (center) and pronounced pseudodecidual stromal change (right), characteristic features of a high-dose progestin response, are seen after therapy. Asterisk, example of PTEN-null glands. PTEN immunohistochemistry with antibody 6H2.1.

future samples, as fully 83% (10 of 12) of those patients having a latent precancer at one time point will have a discernible latent precancer during follow-up (8). As was the case with sampling error, comparison between groups of differently managed patients undergoing similar tissue sampling has controlled this variable.

Lastly, consistently defined hormonal exposures, such as those likely to change endometrial cancer risk, are difficult to achieve outside the controlled setting of a clinical trial. This was a problem in a previous retrospective study that showed a 90% rate of PTEN-null endometrial precancer involution in response to various doses and intervals of medically administered progestins (13). The current study makes use of a systematic approach to therapy to generate more consistent groups for comparison.

This is a study exploring those biological events that are effective during a preclinical phase and have the potential to alter the course of subsequent disease. Ultimately, long-term patient outcomes, including a protracted follow-up interval after withdrawal of therapeutic hormones, are an unmatched gold standard for establishing therapeutic efficacy. We have shown that short-term progestin administration, previously associated with long-term favorable clinical outcomes (cancer risk reduction), can increase the rate of involution of latent precancers while on therapy. This provides additional evidence that the preclinical phases of carcinogenesis have accessible short-term laboratory end points that may be assessed to evaluate the effect of a variety of cancer prevention therapies.

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