DNA Ploidy, Cell Cycle Kinetics, and Low Versus High Grade Atypia in Endometrial Hyperplasia

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Endometrial hyperplasia is an estrogen-dependent lesion that is further classified as simple, complex, or atypical depending on the degree of gland proliferation and the presence or absence of cytologic atypia. Kurman and colleagues' demonstrated a low risk of progression to endometrial carcinoma in patients with simple or complex hyperplasia without atypia (1% and 3%, respectively). The risk of concurrent or subsequent carcinoma increased to 8% in patients with simple hyperplasia with cytologic atypia and 29% in patients with complex hyperplasia with atypia. Most patients who develop endometrial carcinoma in the setting of endometrial hyperplasia have relatively superficial carcinomas of low architectural grade that are associated with a favorable prognosis.2,3 These neoplasms are thought to have significant epidemiologic and prognostic differences from the more poorly differentiated adenocarcinomas arising from atrophic endometrium in older women. The latter tumors are not related to unopposed estrogen stimulation, have a higher architectural grade, are more deeply invasive, and have a less favorable prognosis.

Flow cytometric studies of endometrial adenocarcinoma have demonstrated that DNA aneuploidy and high proliferative activity are poor prognostic factors in endometrial carcinoma.4-8 However, there have been very few studies of DNA ploidy and cell cycle kinetics in endometrial hyperplasia. Existing studies are small and are not accompanied by statistical evaluation of data.

We have performed a flow cytometric analysis of DNA-ploidy, S-phase fraction, and proliferative fraction (S+G2M) in normal proliferative endometrium, simple, complex and atypical endometrial hyperplasia have relatively superficial carcinomas of low architectural grade that are associated with a favorable prognosis.2,3 These neoplasms are thought to have significant epidemiologic and prognostic differences from the more poorly differentiated adenocarcinomas arising from atrophic endometrium in older women. The latter tumors are not related to unopposed estrogen stimulation, have a higher architectural grade, are more deeply invasive, and have a less favorable prognosis.

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We have performed a flow cytometric analysis of DNA-ploidy, S-phase fraction, and proliferative fraction (S+G2M) in normal proliferative endometrium, simple, complex and atypical endometrial hyperplasia and well, moderately, and poorly differentiated endometrial adenocarcinoma. We studied whether flow cytometric parameters distinguish different categories of endometrial hyperplasia and reflect the increased risk of endometrial adenocarcinoma in atypical hyperplasia. We also evaluated whether those hyperplasias proceeding to carcinoma could be identified by DNA ploidy or cell cycle kinetic studies. In addition, we investigated the significance of the degree of cytologic atypia in endometrial hyperplasia related to the incidence of endometrial carcinoma. A previous study1 has found no prognostic value in evaluating the amount of atypia in these lesions.

Finally, we studied whether low grade carcinomas of the type associated with unopposed estrogen stimulation shows differences in cell cycle kinetics and DNA ploidy.

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from the more aggressive carcinomas not usually arising from estrogen dependent lesions. Although endometrial carcinoma previously has been studied by flow cytometry, we thought that these data could prove useful in comparison with our studies of endometrial hyperplasia and could further support the concept that there are two fundamentally different types of endometrial carcinoma.

MATERIALS AND METHODS

The files of the Indiana University Hospital Division of Surgical Pathology were searched for cases coded as proliferative endometrium, any type of endometrial hyperplasia, and endometrial carcinoma between mid-1989 (the beginning of computerized files) and mid-1993, when this study began. Because of the referral nature of our patient base, many cases were excluded because no paraffin blocks were available. All cases of endometrial hyperplasia having paraffin blocks with adequate tissue for flow cytometry were included in this study. In addition, 10 cases of normal proliferative endometrium and 10 to 12 cases each of well, moderately, and poorly differentiated adenocarcinoma with adequate paraffin-embedded tissue were included.

All slides were reviewed by a pathologist (HM) before inclusion in the study. The first 10 proliferative endometrium specimens with adequate material for the study were used. All endometrial hyperplasias were reviewed independently by two pathologists (HM, LMR) and classified as simple or complex hyperplasia with or without atypia by the criteria of Kurman and colleagues. Simple hyperplasia was characterized by glands with irregular contours and focal outpouchings that were no more than moderately crowded. Complex hyperplasia displayed a greater degree of gland budding and branching and more gland crowding with scant stroma separating the glands. Atypical hyperplasia included all cases with nuclear enlargement and loss of polarity.

In addition, atypia was further designated low or high grade based on the degree of nuclear atypia, stratification and loss of polarity (Fig. 1). A consensus diagnosis was reached in all cases. Endometrial carcinomas were classified as well, moderately, or poorly differentiated according to the amount of gland formation and nuclear atypia using standard criteria. All special types (eg, serous, clear cell, etc.) of carcinoma were excluded from this study. Only endometrioid neoplasms were included.

Areas of interest in all slides were indicated with a black marking pen and all paraffin blocks were then scored accordingly. All lesions used for this study were of a size that was believed to ensure their presence in deeper sections. There were no extremely small or focal lesions. Three to five 50 μ sections were cut from the scored area of each block, placed in glass tubes and processed using a modified Hedley technique with prolonged rehydration in deionized water. Sections were deparaffinized using two changes of AmeriClear (Baxter Scientific Products, McGraw Park, IL), and rehydrated with graded alcohols (100%, 95%, 70%, and 50% ethanol). They were then washed twice with distilled water and stored 24 to 48 hours in distilled water. Sections were then minced with scissors and digested with 0.5% pepsin (Sigma, St. Louis, MO) at 35 °C with vortexing every 5 minutes to promote release of nuclei. Enzymatic digestion was stopped by dilution with buffer solution. The suspension was centrifuged at 800 g for 10 minutes. Cell pellets were...
resuspended in buffer and filtered through a 40 μ nylon mesh. Cell suspensions were adjusted to 1–2 x 10⁶ cells/mL. Cells were treated with Triton X-100 followed by incubation with RNase (180 V/mL; Worthington Biochemical, Freehold, NJ) at 37 °C for 20 minutes. Cell pellets were stained with propidium iodide (50 μg/mL in phosphate buffered saline, Sigma) for 24 hours at 4 °C and analyzed on an Epics Profile flow cytometer (Coulter, Hialeah, FL) by gating on peak versus integrated signal. For each sample, 20,000 to 50,000 events were evaluated.

Cases were acceptable for analysis if the coefficient of variation (CV) of the G₀/G₁ peak was <7.0. DNA histograms cell-cycle analysis was performed using Multicycle software (Phoenix Flow System, San Diego, CA). Diploid histograms displayed a single G₀/G₁ peak. Aneuploid histograms displayed two distinct G₀/G₁ peaks with a DNA index of greater than 1.15. A tumor was classified as tetraploid only if a large peak in the G2M region (DI of 1.80-2.2) comprised >20% of the total curve and was associated with a corresponding peak at 8N.

Clinical data were obtained from our files. We were interested primarily in the histologically documented occurrence of endometrial carcinoma in patients with endometrial hyperplasia. For follow-up, we looked only at cases with slides at our institution to ensure consistency of diagnoses. Outside diagnoses of carcinoma are sometimes lesions considered by us to be atypical hyperplasia. No follow-up data were obtained for patients with proliferative endometria or endometrial carcinoma because these cases were included only for comparison of flow cytometric parameters with those of endometrial hyperplasia.

All statistical analyses were performed by the division of Medical Biostatistics at the Indiana University Medical Center. Because these data did not show a normal distribution, they were square root-transformed. S-phase fractions and proliferative fractions (S+G2M) were then compared using Fisher's least significant difference (LSD) test with a global P = .05. Fisher's exact test was used to evaluate the relationship between degree of atypia in hyperplasia and occurrence of endometrial carcinoma.

RESULTS

After excluding cases with insufficient tissue for flow cytometry (referral cases with no paraffin blocks) and those with an unacceptable coefficient of variation (7 cases), we analyzed 8 proliferative endometria, 7 simple endometrial hyperplasias without atypia, 6 complex endometrial hyperplasias without atypia, 26 atypical endometrial hyperplasias, 7 well-differentiated adenocarcinomas, 10 moderately differentiated adenocarcinomas, and 9 poorly differentiated adenocarcinomas. Flow cytometric results and evaluation of degree of atypia and occurrence of carcinoma are listed in Tables 1 and 2.

All proliferative endometria, all endometrial hyperplasias of all types, and all well and moderately differentiated adenocarcinomas in this series were diploid lesions. DNA ploidy studies did not distinguish the different types of endometrial hyperplasia, did not identify the atypical hyperplasias associated with concurrent or subsequent carcinoma, and did not distinguish endometrial hyperplasia from either proliferative endometrium or from well or moderately differentiated adenocarcinoma. However, six of the nine cases of poorly differentiated endometrial adenocarcinoma were aneuploid, a result consistent with data previously reported. Harmon panels of specimens with diploid and aneuploid DNA content are shown in Figure 2.

Analysis of both S-phase fraction and proliferative fraction (S+G2M) yielded similar results. Both S-phase and S+G2M were lowest in proliferative endometria (mean S-phase = 3.86, range 0.6–9.4) Well-differentiated adenocarcinoma (mean S-phase=4.99, range 2.2-8.0) and moderately differentiated adenocarcinoma (mean S-phase=6.46, range 0–11.4) had slightly higher S-phase fractions, but statistical analysis showed no significant difference (P = .05) in S-phase or S+G2M for proliferative endometrium, well or moderately differentiated adenocarcinoma.

There were also no significant differences between S-phase fractions or S+G2M for simple hyperplasia (mean S-phase = 12.98, range 8.8–22.6), complex hyperplasia (mean S-phase = 10.7, range 8.8–14.2) or atypical hyperplasia (mean S-phase = 11.04, range 3.8–27.0). However, all types of hyperplasia had significantly higher (P = .05) S-phase fractions and proliferative fractions (S+G2M) than did proliferative endometria or well- or moderately differentiated adenocarcinomas. There were no significant differences between S-phase or S+G2M of atypical hyperplasias that did not progress to carcinoma compared with those that did progress to carcinoma.

Poorly differentiated adenocarcinomas had significantly higher (P = .05) S-phase fractions (mean S-phase = 19.07, range 6.4–35.1) and proliferative fractions (S+G2M) than all endometrial hyperplasias, proliferative endometria and well- and moderately differentiated adenocarcinomas.

Clinical data for patients with endometrial hyperplasia are presented in Table 1. Follow-up data were available for eight patients whose endometrial hyperplasia showed high grade atypia. Three of the eight had concurrent or
TABLE 1. CELL CYCLE PARAMETERS AND OCCURRENCE OF CARCINOMA IN ENDOMETRIAL HYPERPLASIA

<table>
<thead>
<tr>
<th>Simple Hyperplasia (All Diploid)</th>
<th>Complex Hyperplasia (All Diploid)</th>
<th>Atypical Hyperplasia (All Diploid)</th>
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<tr>
<td><strong>S-Phase</strong></td>
<td><strong>S + G2M</strong></td>
<td><strong>S-Phase</strong></td>
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<tr>
<td><em>(mean = 11.6)</em></td>
<td><em>(mean = 14.53)</em></td>
<td><em>(mean = 10.7)</em></td>
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<td>9.4</td>
<td>11.4</td>
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<td>20.6</td>
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NA = not available.
subsequent carcinoma. Follow-up was available in 13 patients with endometrial hyperplasia displaying low grade cytologic atypia. Four of the 13 developed carcinoma. The number of women with complex hyperplasia with atypia progressing to carcinoma was greater than the number of women with simple hyperplasia with atypia who developed carcinoma, but this difference was not statistically significant. In addition, although the presence of cytologic atypia was a significant adverse prognostic feature in endometrial hyperplasia, the degree of epithelial stratification, mitotic activity, and cytologic atypia were not significantly associated with progression to carcinoma.

Endometrial adenocarcinoma has been well studied by flow cytometry. DNA aneuploidy has been associated with high architectural and cytologic tumor grade, adverse histologic types, deep myometrial invasion, high tumor stage, and absence of hormone receptors. An aneuploid is also an independent adverse prognostic factor in endometrial adenocarcinoma, and high S-phase fractions are associated with higher tumor grade and increased mortality.

There have been few studies of DNA ploidy and cell cycle kinetics in endometrial hyperplasia. Existing studies are small, the results are conflicting, and often no statistical correlation of data is presented. An early evaluation of DNA content in atypical endometrial hyperplasia by Katayama and Jones (using a flame-dry method) found some aneuploid cells in a single case, but the criteria for the diagnosis of atypical hyperplasia were not given. Microspectrophotometric measurements of Feulgen-stained nuclei by Wagner and colleagues displayed aneuploidy in 2 of 10 cases of adenomatous hyperplasia. Ferency, using *in vitro* DNA histoaautoradiography, found increased S-phase duration proceeding from endometrial hyperplasia to endometrial carcinoma, sug-

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<tr>
<td><strong>Proliferative EM</strong> (All Diploid)</td>
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<td>S-Phase</td>
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DISCUSSION

Endometrial hyperplasia has long been recognized as a potential precursor to endometrial adenocarcinoma. Tavassoli and Kraus demonstrated a significant incidence of endometrial adenocarcinoma in uteri resected for atypical endometrial hyperplasia. More recently, Kurman and colleagues demonstrated that cytologic atypia in hyperplasia is more significantly related to the development of carcinoma than is the architectural complexity seen in endometrial hyperplasia. These authors found that only 1.6% of 122 patients with endometrial hyperplasia without atypia progressed to carcinoma, whereas 23% of women whose hyperplasia displayed cytologic atypia developed endometrial carcinoma. The number of women with complex hyperplasia with atypia progressing to carcinoma was greater than the number of women with simple hyperplasia with atypia who developed carcinoma, but this difference was not statistically significant. In addition, although the presence of cytologic atypia was a significant adverse prognostic feature in endometrial hyperplasia, the degree of epithelial stratification, mitotic activity, and cytologic atypia were not significantly associated with progression to carcinoma.

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Endometrial Hyperplasia

Evaluation of DNA ploidy and cell cycle kinetics was facilitated by development of flow cytometry. The first flow cytometric study of endometrial hyperplasia was that of Feichter and colleagues. These authors reported that all endometrial hyperplasias and well-differentiated adenocarcinomas were diploid lesions, whereas 60% of their poorly differentiated adenocarcinomas were aneuploid. S-phase fractions were said to increase in proportion to the degree of hyperplastic change, although adenomatous hyperplasia and well-differentiated adenocarcinoma were indistinguishable by flow cytometry. Poorly differentiated adenocarcinomas were characterized by higher S-phase fractions and frequent aneuploidy. This series displayed small sample numbers and no statistical evaluation was presented. Thornton and coworkers later used flow cytometry to study DNA ploidy and proliferative indices of normal, hyperplastic, and malignant human endometrium. Aneuploidy was demonstrated in one case of cystic hyperplasia and two cases of hyperplasia with architectural atypia. The authors noted considerable overlap, but believed that a trend existed showing higher proliferative indices in atypical than in nonatypical hyperplasia and in less differentiated than better differentiated carcinomas. They postulated that aneuploid cases of endometrial hyperplasia could represent lesions with increased malignant potential. Subsequently, Lindahl and Alm found aneuploidy in 6% of normal endometria, 20% of endometrial hyperplasias and 29% of well-differentiated adenocarcinomas. Increasing severity of hyperplasia displayed increased S-phase fractions. No statistical analysis of data is presented. Most recently, Punnonen and colleagues found no aneuploid in 22 cases of endometrial hyperplasia. S-phase fractions were very slightly higher in atypical hyperplasia than in nonatypical hyperplasia, but there was no significant difference in the rate of cell proliferation between grade 1 adenocarcinomas and hyperplasias with or without atypia. The only significant difference in S-phase fractions was between grade 1 and grade 2-3 carcinomas.

Thus, the relatively few previous studies of DNA ploidy and cell cycle kinetics in endometrial hyperplasia have yielded inconsistent, conflicting results. In contrast to several previous studies and in consistency with others, our study of 13 cases of nonatypical endometrial hyperplasia and 26 cases of atypical hyperplasia showed all cases to be diploid. DNA diploidy was not useful in separating hyperplastic endometrium from either benign proliferative endometrium or low grade adenocarcinoma. It was also not a useful parameter in distinguishing the different types of endometrial hyperplasia (simple, complex or atypical). In contrast to the study of Thornton and colleagues, DNA ploidy in our experience was not useful in predicting which atypical hyperplasias progressed to carcinoma, probably because carcinomas arising in atypical hyperplasia are usually well differentiated. These carcinomas are most often diploid lesions both in our experience and in other studies. In contrast, two-thirds of the poorly differentiated endometrial adenocarcinomas in our study were aneuploid lesions. Previous studies have also associated aneuploidy with high tumor grade.
We were unable to confirm the significance of cell cycle kinetic studies in endometrial hyperplasia previously suggested by some authors.14-17 This disparity may be explained at least in part by the use of more modern technology, larger sample sizes than in some prior studies, and use of a thorough statistical evaluation. S-phase fraction and proliferative fraction (S+G2M) in our study showed no significant differences in any types of endometrial hyperplasia (simple, complex, or atypical) and did not distinguish atypical hyperplasia progressing to carcinoma from atypical hyperplasia not progressing to carcinoma. However, all hyperplasias displayed significantly higher S-phase and proliferative fractions than proliferative endometrium, well or moderately differentiated adenocarcinoma. The reason for greater cell proliferation in endometrial hyperplasia than in well-differentiated adenocarcinoma is not clear. As expected, poorly differentiated adenocarcinomas displayed significantly higher S-phase fractions and proliferative fractions than all other lesions studied.

The results of our study concur with previous findings of Kurman and colleagues1 that evaluation of degree of cytologic atypia in endometrial hyperplasia does not aid in predicting risk of progression to endometrial carcinoma. We found no significant difference in occurrence of carcinoma in hyperplasia with low versus high grade atypia.

The fact that all endometrial hyperplasias and well-to-moderately differentiated adenocarcinomas in our study were diploid lesions, whereas poorly differentiated carcinomas were often aneuploid tumors with significantly higher S-phase and proliferative fractions than both hyperplasias and lower grade carcinomas adds further support to the theory that there are two fundamentally different types of endometrial carcinoma. Bohman2 and Deligdisch and Cohen3 postulated that well- and moderately differentiated endometrial adenocarcinomas arise in a milieu of excessive estrogen stimulation and are related to endometrial hyperplasia. Poorly differentiated carcinomas are not hormone dependent, do not usually arise in endometrial hyperplasia, are more deeply invasive, and have a much worse prognosis.

In summary, all endometrial hyperplasias and low grade endometrial carcinomas in our study were diploid lesions. S-phase fractions and proliferative fractions did not distinguish different types of hyperplasia and did not predict which cases of atypical hyperplasia were associated with endometrial carcinoma. Degree of cytologic atypia (low versus high) was not significantly predictive of the likelihood of atypical hyperplasia being associated with carcinoma.

REFERENCES