Expression of Metastases-associated Genes in Cervical Cancers Resected in the Proliferative and Secretory Phases of the Menstrual Cycle

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ABSTRACT

Previous retrospective studies suggest that the phase of the menstrual cycle at surgery (proliferative versus secretory) for breast cancer may significantly affect patient survival. Fluctuations during the menstrual cycle of the expression of genes involved in metastases in breast cancer tissue have also been reported. We hypothesized that the menstrual phase may also affect similar changes in gene expression of other cancers. We focused our attention on cancer of the uterine cervix because the hysterectomy specimen obtained at original surgery for the cancer can be used retrospectively to determine cycle phase. We analyzed tumor specimens from 36 premenopausal cervical cancer patients who had undergone hysterectomy as their primary treatment. We used reverse transcription-PCR to quantify gene expression during the different phases of the menstrual cycle as determined from the endometrial specimen. We explored a panel of genes that may affect metastatic propensity, namely, metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase-2 (TIMP-2), cyclooxygenase 1 and 2 (COX-1 and COX-2), and vascular endothelial growth factor (VEGF). A significantly higher level of TIMP-2 and COX-2 gene expression ($P = 0.007$ and 0.030, respectively) was detected during the proliferative phase compared to the secretory phase of the cycle. The expression of the other genes was not significantly affected by the stage of the menstrual cycle. The finding that TIMP-2 and COX-2 expression in cervical cancer may be affected by the stage of the menstrual cycle supports the hypothesis that ovarian hormones may affect the expression of genes involved in metastasis. These findings need to be replicated, and their implications for tumor angiogenesis, invasion, and metastatic propensity need to be explored both in human studies and in experimental models.

INTRODUCTION

Numerous studies have suggested that survival among premenopausal breast cancer patients without distant metastases may be affected by the hormonal milieu at the time of surgery, especially in women found to have axillary lymph node involvement (1–8). Women undergoing surgery during the proliferative phase tend to have worse survival than those operated on during the secretory phase. Several biological mechanisms to account for this have been suggested (9–11).

These include cyclical patterns of immune function, as well as aspects of cancer cell division and apoptosis, that may be affected by the hormonal fluctuations of the menstrual cycle. If genes involved in various aspects of carcinogenesis were hormonally regulated, their up-regulation at specific times in the menstrual cycle might predispose seeding cancer cells toward better survival, faster growth rate, or increased metastatic potential. To our knowledge, two molecular studies have been published to date on this subject. The first study, reported in the Lancet by Saad et al. (9), used Northern blot analysis of RNA to measure the expressions of a number of metastases-related genes, namely, cathepsin-L, MMP-9,2 MMP-2, TIMP-1, TIMP-2, and TP53, in breast cancer specimens from 27 premenopausal women. They classified the specimens in terms of menstrual cycle phase by serum levels of estradiol and progesterone on the day of operation. They found that cathepsin L, MMP-9, and wild-type p53 expressions were significantly elevated in breast tumors resected during the phases of the menstrual cycle in which estradiol was $\geq 20$ nmol/liter and progesterone was low, i.e., on days in which a proliferative endometrium should be present. More recently, in a study of 198 breast cancer patients, Balsari et al. (11) identified a statistically significant increase of HER2neu protein expression in tumors from women operated on during the follicular phase of the menstrual cycle, suggesting that overexpression of the HER2 gene may explain the worse outcome for patients who are operated on during such a phase.

We hypothesized that a similar phenomenon could be occurring in other tumors, and we selected invasive cervical cancer for study because in premenopausal patients initially treated by hysterectomy, the phase of the cycle (proliferative

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2 The abbreviations used are: MMP, matrix metalloproteinase; RT-PCR, reverse transcription-PCR; TIMP, tissue inhibitor of metalloproteinase; COX, cyclooxygenase; VEGF, vascular endothelial growth factor; CI, confidence interval.
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We also investigated COX-1 as a “control” for the reported fluctuations in COX-2 with estrus in mice and sheep (16, 17). Noticeably, a large range of measured gene expression was observed. For VEGF expression, sufficient mRNA was available in only 18 of 36 specimens. The other markers were tested in all 36 patients. The variables of interest (i.e., the ratios of expression of the genes of interest to that of β-actin) were not normally distributed. Logarithmic values were therefore taken for statistical analysis. Differences between the proliferative and secretory specimens were tested for by Student’s t test, and the statistical significance levels were also computed using the Mann-Whitney nonparametric two-sample approach. The means calculated (and quoted in Table 1, see “Results”) are geometric means with associated 95% CIs obtained by exponentiating the means and 95% CIs of the logarithmically transformed values. All statistical significance levels (Ps) quoted are two-sided. There was little difference between any of the Ps calculated from the t tests and from the nonparametric approach, and the values quoted are from the t tests.

**RESULTS**

The phase of the menstrual cycle at the time of surgery was based on endometrial dating: (a) in 21 of 36 patients, the tumor specimens were removed during the proliferative phase; (b) in 12 of 36 patients, the tumor specimens were removed during the secretory phase; and (c) in 3 of 36 patients, the tumor specimens were removed during the menstrual phase. Table 1 summarizes the results of the study. The geometric means of TIMP-2 and COX-2 gene expression are approximately 3-to 3.5-fold greater in the cervical cancer tissues removed during the proliferative phase of the cycle compared with cancer tissues removed during the secretory phase (P = 0.007 and 0.030, respectively). The expression of MMP-9, VEGF, and COX-1 was similar in the two groups. Figs. 1–5 show the individual data point for the results for each gene measured, plotted against the timing of surgery as determined by pathological dating of the matched endometrial tissue.

Noticeably, a large range of measured gene expression values was observed, ranging from a low of 0.27 to a high of 4.71. This indicates the variability in gene expression levels across different samples and may be indicative of the complexity and diversity of gene expression patterns in cervical cancer.

**Table 1** Comparison of gene expressiona in cervical cancers removed during the proliferative (P) and secretory (S) phases of the menstrual cycle

<table>
<thead>
<tr>
<th>Gene</th>
<th>Geometric mean for P</th>
<th>Geometric mean for S</th>
<th>P:S</th>
<th>95% CI</th>
<th>P (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td>3.13</td>
<td>3.88</td>
<td>0.81</td>
<td>0.27–2.43</td>
<td>0.70</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>53.5</td>
<td>16.9</td>
<td>3.17</td>
<td>1.43–7.02</td>
<td>0.007</td>
</tr>
<tr>
<td>VEGF</td>
<td>1.3</td>
<td>0.9</td>
<td>1.4</td>
<td>0.37–5.60</td>
<td>0.61</td>
</tr>
<tr>
<td>COX-2</td>
<td>1984.8</td>
<td>587.3</td>
<td>3.4</td>
<td>1.17–3.38</td>
<td>0.030</td>
</tr>
<tr>
<td>COX-1</td>
<td>14</td>
<td>10</td>
<td>1.4</td>
<td>0.41–4.71</td>
<td>0.60</td>
</tr>
</tbody>
</table>

a Ratios of PCR products of gene of interest to β-actin.

versus secretory versus menstrual) can be identified by direct microscopic inspection of the endometrium. We have investigated fluctuations in the expression of two of the genes studied by Saad et al. (9), namely, MMP-9 and TIMP-2, in premenopausal cervical cancer specimens. We have also investigated fluctuations in COX-2 and VEGF because of their emerging central role as regulatory molecules for tumor angiogenesis, metastatic propensity, and cell adhesion (12–15) and because of the reported fluctuations in COX-2 with estrus in mice and sheep (16, 17). We also investigated COX-1 as a “control” molecule (18).

**MATERIALS AND METHODS**

We identified 38 frozen cervical cancer specimens from premenopausal patients treated by hysterectomy at Los Angeles County/University of Southern California Medical Center. All women had undergone hysterectomy as the primary treatment for stage IB-IIA cervical cancer. In 36 of 38 cases, the original pathological specimen of the uterus was available for review to describe the phase of the menstrual cycle. Their endometrial phase was classified as proliferative or secretory by an experienced pathologist (J. F.).

Total mRNA was isolated from 36 cervical cancer specimens. mRNA was isolated from human biopsy tissues using the guanidinium isothiocyanate method with the Quickprep Micro mRNA Purification Kit (Amersham Pharmacia Biotech). Briefly, biopsy tissues were snap-frozen in liquid nitrogen and pulverized with a mortar and pestle. The pulverized tissue sample was then homogenized using a guanidinium isothiocyanate solution. mRNA isolation and purification were carried out according to instructions provided in the kit. The isolated mRNA was then dissolved in 60 μl of diethyl pyrocarbonate-treated water. The mRNA was converted to cDNA, and quantitative RT-PCR (19) was performed to determine the gene expressions of MMP-9, TIMP-2, VEGF, COX-2, and COX-1. RT-PCR was performed using a fluorescence detection method with the TaqMan (ABI PRISM 7700 Sequence Detection System; Perkin-Elmer Applied Biosystems). This method uses a dual-labeled fluorogenic probe that anneals specifically between the forward and reverse primers. The fluorogenic probes are labeled with a quencher dye (TAMRA) at the 3’ end and a reporter dye (6FAM) at the 5’ end. Laser stimulation within the capped wells containing the reaction mixture causes emission of the quencher dye until the probe is cleaved by the 5’→3’ exonuclease activity of DNA polymerase during the extension phase of PCR. An amplicon is produced simultaneously when cleavage of the quencher dye causes release of the reporter dye, which results in the emission of a fluorescent signal that is detected by the TaqMan detection camera. The amount of signal produced at a threshold cycle within the purely exponential phase of the PCR reaction reflects the starting copy number of the sequence of interest. The gene expression data are expressed as a ratio between the PCR products formed by the gene of interest relative to that formed by β-actin, the internal reference gene (18). For each tumor specimen, each gene expression value was assigned to the phase of the cycle determined by endometrial dating (proliferative versus secretory versus menstrual).

For each marker, gene expression measurements at RT-PCR were repeated at least two times.
values were observed for each of the markers (gene of interest: β-actin). In the case of COX-2, the values varied from 47 to 91,269, a 1,936-fold range. However, within the secretory phase, only a 260-fold range was observed (47–9,790). For TIMP-2, the range of gene expression was 1.29–1,252, a 960-fold range. For MMP-9, the range of gene expression was 0.07–119, a 1,700-fold range. For COX-1, the range of gene expression was 1–1,897, a 1,897 fold range. For the 18 patients whose specimens had sufficient mRNA to conducting and re-
peating the experiments twice, VEGF gene expression ranged from 0.09–25, a 2,700-fold range. Tumors removed during the proliferative phase consistently had a trend for the highest gene expression.

DISCUSSION

Our data demonstrate that in cervical cancer, intratumoral gene expression of COX-2 and TIMP-2 fluctuates significantly during the menstrual cycle, with highest values in the proliferative phase of the endometrium. This is consistent with the data reported by Saad (9) and Balsari (11) on breast cancer, who found that other markers for tumor aggressiveness and invasion such as cathepsin D and HER2 tend to be overexpressed during the follicular phase (i.e., proliferative phase of the endometrium).

It is intriguing to notice that we found the same trend for COX-2 and TIMP-2 in cervical cancer. Although the geometric means of the values were significantly different for TIMP-2 and COX-2 gene expression during the different phases of the cycle, considerable overlap of values during the proliferative and secretory phase was noticed for all markers studied. This is to be expected if gene expression reflects the physiological hormonal fluctuations during the menstrual cycle because circulating estradiol concentrations peak during both the secretory and proliferative phase of the endometrium. In the absence of blood specimens to document the exact hormonal content at the time of surgery, it is impossible to correlate gene expression with hormonal measurements and precise dating. Future studies with a much larger sample of patients and concurrent blood hormonal levels are likely to better identify which window of time during the proliferative phase is associated with increased intratumoral markers of metastatic potential.

This is the first report of menstrual cycle-associated
COX-2 fluctuations in humans, although fluctuation has been observed previously in animals. COX-2 determination during the different phases of the estrus cycle in a rat model showed COX-2 overexpression at the peak of the serum estradiol level that precedes ovulation (17). COX-2 was found to be highly and transiently expressed in sheep 12–15 days after the estrous cycle and declined thereafter to undetectable levels (16). In contrast, COX-1 expression did not change significantly during the menstrual cycle in either the animal studies or the present study, which is consistent with previous studies showing its expression to be constitutive and noninducible (18).

COX-2 is often found to be elevated in cancers of major concern and has received much attention in recent years because of the possibility that tumors might be prevented by its inhibition. Investigations into the role of COX-2 in tumorigenesis have disclosed that it promotes angiogenesis and cell adhesion and inhibits apoptosis (13–15). These findings suggest several mechanisms by which higher expression of COX-2 could be a risk factor at the time of surgery for cervical cancer. For example, cervical cancer surgery carried out during periods of high COX-2 expression might enhance the adhesion properties and thus enhance the probability of survival of cancer cells that detach from the tumor during the surgery. Similarly, a decreased propensity for apoptosis would help the survival of cells that detach from the tumor.

If it is shown that survival of cervical cancers patients is related to COX-2 expression, it may also be possible to eliminate this risk by treating patients with COX-2 inhibitors before surgery.

Similar to the findings reported by Balsari et al. (11) and Saad et al. (9), our results should only be considered exploratory, requiring confirmation and extensive studies to elucidate possible mechanisms and the importance of the findings. However, it is intriguing to think that in premenopausal women, cyclic hormonal fluctuation might potentially affect the behavior of cancer cells when tumor surgery is performed. We have begun a tumor registry-based retrospective study of survival of premenopausal cervical cancer patients to investigate whether the phase of the menstrual cycle at operation has affected patient survival. Much more valuable would be a prospective study with determination of COX-2 and other gene expressions as well as...
precise hormonal determinations at the time of operation to be correlated with subsequent survival.

Finally, it could also be speculated that hormonal changes during the follicular phase of the menstrual cycle may affect the biology of tumors other than breast or cervical cancer in pre-menopausal women: if such differences at the time of surgery prove to reflect on patient outcome, it could become crucial to correctly schedule any tumor surgery in premenopausal women.

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