The Effects of Tamoxifen on Endometrial Insulin-Like Growth Factor-1 Expression

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Objective: To determine whether modulation of insulin-like growth factor-1 and insulin-like growth factor–binding protein-1 expression underlies the uterotrophic effects associated with tamoxifen therapy in postmenopausal breast cancer patients.

Methods: Using immunohistochemical techniques, we analyzed 37 endometrial specimens from biopsies (n = 18) or hysterectomies (n = 19) for Ki-67, insulin-like growth factor-1, and insulin-like growth factor–binding protein-1 expression. Specifically, five secretory- and three proliferative-phase endometrial specimens were used as controls; 20 specimens (including two endometrial adenocarcinomas) were analyzed from postmenopausal breast cancer patients treated with tamoxifen (20 mg/day) for at least 6 months; and nine endometrial adenocarcinoma specimens from patients not treated with tamoxifen were studied. Intensity of immunostaining was quantified using digitized imaging techniques.

Results: Insulin-like growth factor-1 and insulin-like growth factor-1–binding protein-1 were found to be expressed in normal and neoplastic endometrium of all patients, regardless of tamoxifen treatment. However, insulin-like growth factor-1 expression varied cyclically in histologically normal endometrium, was reduced in undifferentiated endometrial tumors, and was upregulated in tamoxifen-treated specimens. Insulin-like growth factor–binding protein-1 immunostaining did not vary during the menstrual cycle, but it was reduced significantly in benign tamoxifen-exposed tissue and endometrial adenocarcinomas, regardless of degree of differentiation or tamoxifen exposure. No correlation was found between the expression of insulin-like growth factor-1 and insulin-like growth factor–binding protein-1 and the proliferative indices of the tissues examined.

Conclusion: The expression of insulin-like growth factor-1 and insulin-like growth factor–binding protein-1 in the uterus supports an autocrine and/or paracrine role for these proteins in endometrial physiology. Although further studies are needed, our investigation suggests that altered expression of insulin-like growth factor-1 and insulin-like growth factor–binding protein-1 may contribute to the uterotrophic effects of tamoxifen. (Obstet Gynecol 1998;91:45–50.)

Tamoxifen is a nonsteroidal triphenylethylene antiestrogen approved by the Food and Drug Administration in 1977 as an adjunctive therapy for postmenopausal women with advanced breast cancer. Since approval, large clinical trials have shown tamoxifen to improve the recurrence-free interval and overall survival in both premenopausal and postmenopausal women with breast cancer. These trials have provided the basis for the initiation of studies in the United States and Europe to test the efficacy of tamoxifen as a chemopreventive agent in healthy premenopausal and postmenopausal women at high risk for developing breast cancer.

Tamoxifen has long been considered a safe medication with few serious side effects; however, recent reports have cited an increase in endometrial cancer occurring in patients using the drug. In 1994, the National Surgical Adjuvant Breast and Bowel Project B-14 trial1 revealed a 7.5-fold increase in the risk of developing endometrial cancer in women using tamoxifen compared with placebo. Despite the clear benefit of tamoxifen in preventing recurrent breast cancer, the broadening indications for the drug to include prophylaxis bring into question whether such side effects are...
tolerable to an otherwise healthy patient. Clearly, there is an impetus to elucidate the mechanism of tamoxifen-induced endometrial carcinogenesis in order to better understand and predict which group of patients would be at higher risk for endometrial cancer and thus warrant increased surveillance.

The biologic effects of insulin-like growth factor-1 are mediated by a complex system that includes growth factors, membrane tyrosine kinase receptors, and binding proteins. Animal research has demonstrated that 17β-estradiol modulates uterine expression of proteins within the insulin-like growth factor system. In addition, many animal and human uterine cell types contain functional receptors for insulin-like growth factor-1 and respond mitogenically to insulin-like growth factor-1 in vitro. These studies have led to the conclusion that insulin-like growth factor-1 is a mediator of estrogen-stimulated uterine proliferation. Furthermore, the uterotrophic effects of tamoxifen, like those of estrogen, have also been shown to involve the upregulation of insulin-like growth factor-1 in animal studies. This leads to the hypothesis that the effect of tamoxifen on the human endometrium may also be due, at least in part, to the regulation of genes in the insulin-like growth factor pathway.

The aim of this preliminary study was to determine if tamoxifen therapy modulated the expression of insulin-like growth factor-1 and insulin-like growth factor-binding protein-1 by comparing the immunohistochemical staining intensity of benign and neoplastic human endometrium with that of endometrium obtained from tamoxifen-treated patients.

### Materials and Methods

This study was carried out under a protocol approved by the Clinical Investigation Committee and the Human Use Committee of Walter Reed Army Medical Center. Thirty-seven endometrial tissue specimens obtained from endometrial biopsies (n = 18) and hysterectomies (n = 19) were analyzed. Eighteen biopsy specimens were from postmenopausal breast cancer patients treated with tamoxifen (20 mg/day) for at least 6 months before analysis, and two hysterectomy specimens were from patients who developed endometrial adenocarcinoma (grade 1) while using tamoxifen. Nine hysterectomy specimens were from endometrial adenocarcinomas (four grade 1, three grade 2, and two grade 3) from patients not receiving tamoxifen and without a history of breast cancer.

Five secretory phase and three proliferative phase hysterectomy specimens were analyzed as normal controls. These specimens were obtained from premenopausal women undergoing surgery for leiomyomas (n = 4), adenomyosis (n = 2), and benign ovarian cysts (n = 2).

Five-micron sections of paraffin-embedded tissue were placed onto sialinized slides. Previously characterized antibodies against insulin-like growth factor-1 (NIH Repository, Bethesda, MD), insulin-like growth factor-binding protein-1 (Upstate Biotechnology Incorporated, Lake Placid, NY), and Ki-67 (DAKO Corporation, Carpinteria, CA) were used for immunocytochemistry according to the manufacturers’ specifications. Negative controls included omission of the primary antibody and incubation with nonimmune serum. Background immunostaining was found to be negligible when nonimmune serum was substituted for specific antiserum. Insulin-like growth factor-1 specificity was confirmed by preabsorption of the antibody with purified peptide (1 µg of peptide to 1:250 dilution of antibody) overnight at 4°C followed by centrifugation at 100,000 × g before immunostaining. The preabsorption with purified insulin-like growth factor-1 significantly attenuated insulin-like growth factor-1 immunostaining (data not shown). Immunostaining was visualized by the avidin-biotin-peroxidase complex method using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) with 2,2'-diaminobenzidine as a substrate. Hematoxylin was used occasionally to counterstain the nuclei of the specimens after immunostaining.

A pathologist (LH) blinded to the clinical data analyzed and graded all specimens using standard International Federation of Gynecology and Obstetrics guidelines. The proliferative index was expressed as the percentage of nuclei positive for Ki-67 expression per total number of cells counted, with at least 500 epithelial cells evaluated for each specimen, using a 40X objective.

Quantitation of the immunostaining intensity for insulin-like growth factor-1 and insulin-like growth factor-binding protein-1 was performed by image analysis using the Image-Pro Plus Analysis System (Media Cybernetics, Silver Spring, MD). The system consists of an integrated central processing unit, image monitors, a microscope, and a 3-CCD (U-TVIX) video camera (OPELCO, Sterling, VA). Upon calibration of the system to detect immunoreactive cells, the relative optical density per unit area was determined using the Image-Pro Plus software (Media Cybernetics). For each sample, nine separate areas on the specimen were analyzed, and the mean relative optical density values with standard errors were determined. Statistical analysis was carried out using an unpaired two-tailed student t test. P < .05 was considered statistically significant.
Results

Immunohistochemistry was used to evaluate insulin-like growth factor-1 and insulin-like growth factor-binding protein-1 expression and its relation to the proliferative index. In proliferative endometrium, there was very weak glandular and stromal cell immunostaining for insulin-like growth factor-1 (Figure 1A). The level of expression of the insulin-like growth factor-1 protein did not correlate with the number of cycling epithelial cells in the proliferative endometrium, as demonstrated by the immunodetection of the proliferation marker Ki-67 (Figure 1B).

In secretory endometrium, insulin-like growth factor-1 protein expression was more distinct than that observed in the proliferative phase, showing both epithelial and stromal cell immunolocalization (Figure 1C).

Image quantitation demonstrated that secretory epithelium expressed significantly greater levels of insulin-like growth factor-1 protein than proliferative epithelium. As in the proliferative phase, insulin-like growth factor-1 expression during the secretory phase did not correlate with the level of epithelial cell proliferation, but instead was associated with enhanced growth of the stroma, a characteristic of the luteal phase (Figure 1D). Quantitation of the number of epithelial cells expressing the Ki-67 antigen revealed that the mean labeling index was significantly greater in the proliferative endometrium than in the secretory endometrium (Table 1).

Immunolocalization and quantitative analysis of insulin-like growth factor-binding protein-1 did not reveal significant differences in expression during the
Table 1. Immunostaining Intensity and Proliferative Indices for Endometrial Specimens

<table>
<thead>
<tr>
<th>Tissue sample</th>
<th>IGF-1*</th>
<th>IGF binding protein-1*</th>
<th>Proliferative index (%)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferative endometrium</td>
<td>0.054 ± 0.01</td>
<td>2.09 ± 0.13</td>
<td>34 ± 8.0</td>
</tr>
<tr>
<td>Secretory endometrium</td>
<td>0.147 ± 0.001⁴</td>
<td>2.24 ± 0.05</td>
<td>2 ± 0.99⁴</td>
</tr>
<tr>
<td>Benign endometrium from tamoxifen-exposed patients</td>
<td>1.120 ± 0.08⁴</td>
<td>1.43 ± 0.09³</td>
<td>13 ± 2.0³</td>
</tr>
<tr>
<td>Endometrial adenocarcinoma from tamoxifen-exposed patients</td>
<td>0.455 ± 0.06³</td>
<td>1.29 ± 0.19³</td>
<td>25 ± 5.0</td>
</tr>
<tr>
<td>Well-differentiated endometrial adenocarcinomas from patients not exposed to tamoxifen</td>
<td>0.250 ± 0.033³</td>
<td>1.30 ± 0.19³</td>
<td>27 ± 6.0</td>
</tr>
<tr>
<td>Undifferentiated to moderately differentiated endometrial adenocarcinomas from patients not exposed to tamoxifen</td>
<td>0.098 ± 0.013³</td>
<td>1.130 ± 0.19³</td>
<td>36 ± 7.0</td>
</tr>
</tbody>
</table>

IGF = insulin-like growth factor-1.

Data are presented as mean ± standard deviation. Units of relative intensity of immunostaining are expressed as pixel intensity units.

* IGF-1 immunostaining intensity compared with proliferative endometrium.

¹ IGF-binding protein-1 immunostaining intensity compared with proliferative endometrium.

³ Proliferative index is presented as the percentage of endometrial epithelial cells that expressed the proliferation marker Ki-67 (% Ki-67-positive nuclei/total number of cells). Proliferative indices compared with proliferative endometrium.

⁴ P < .01.
⁵ P < .05.

menstrual cycle (Figures 1E and 1F). Insulin-like growth factor–binding protein-1 was expressed equally in epithelial and stromal compartments of the endometrium during the follicular (Figure 1E) and luteal (Figure 1F) phases of the menstrual cycle (Table 1).

Disparate immunostaining of insulin-like growth factor-1 was noted in nine endometrial adenocarcinomas obtained from patients not treated with tamoxifen. Poorly differentiated tumors tended to exhibit lower immunostaining for insulin-like growth factor-1 protein (Figure 1G) compared with well-differentiated tumors (Figure 1H). The staining intensity determined by image quantitation was significantly greater in well-differentiated tumors compared with undifferentiated tumors. However, adenocarcinomas, regardless of degree of differentiation, expressed significantly higher levels of insulin-like growth factor-1 than proliferative endometrium. Well-differentiated tumors demonstrated higher epithelial insulin-like growth factor-1 protein expression than secretory-phase endometrium, but undifferentiated tumors displayed lower levels of immunostaining. The insulin-like growth factor–binding protein-1 staining intensity of both the well- and poorly differentiated adenocarcinomas was significantly less than that exhibited by either proliferative or secretory endometrium. No significant difference was observed when the non–tamoxifen-exposed tumors were compared with each other (Table 1).

Insulin-like growth factor-1 immunostaining also was observed in benign and malignant endometrium of tamoxifen-treated postmenopausal patients (Figures 1I and 1K). Analysis of the relative staining intensities revealed that epithelial insulin-like growth factor-1 expression in tamoxifen-exposed tissue was significantly greater than that in benign and malignant specimens from untreated tissue (Table 1). As shown by the representative micrograph in Figure 1I, benign tissue that had been exposed to tamoxifen demonstrated prominent epithelial insulin-like growth factor-1 expression when compared with either proliferative or secretory endometrium.

Insulin-like growth factor-1 expression in two well-differentiated adenocarcinomas from tamoxifen-treated patients and the well-differentiated tumors of patients not exposed to tamoxifen both demonstrated regions of distinct insulin-like growth factor-1 immunostaining (Figure 1). However, quantitation revealed that the insulin-like growth factor-1 staining intensity in well-differentiated endometrial tumors from patients treated with tamoxifen was significantly greater than that found in well-differentiated tumors from untreated patients (Table 1).

Measurements of insulin-like growth factor–binding protein-1 in benign and malignant tamoxifen-exposed tissue demonstrated lower levels of immunostaining when compared with normal endometrium. Variation was not noted in the level of insulin-like growth factor–binding protein-1 expression between the endometrial adenocarcinomas with regard to tamoxifen exposure. In addition, no correlation was observed between the expression of insulin-like growth factor-1 or insulin-like growth factor–binding protein-1 and the proliferative indices of the malignancies, regardless of tamoxifen exposure (Table 1).

Like the endometrium, the myometrium of all specimens showed consistent immunostaining for insulin-like growth factor-1 and insulin-like growth factor–binding protein-1. Subjectively, myometrial insulin-like growth factor-1 expression exhibited a cyclic expression pattern similar to the pattern of the control endome-
trium (Figures 1L and 1M). In addition, the expression of insulin-like growth factor-1 in the myometrium of tamoxifen-treated patients resembled myometrial expression during the secretory phase (Figure 1N). Insulin-like growth factor-binding protein-1 expression did not appear to be altered by tamoxifen treatment (data not shown).

Discussion

This study investigated the cellular distribution of insulin-like growth factor-1 and insulin-like growth factor-binding protein-1 expression and their association with cell proliferation in the human endometrium. Both insulin-like growth factor-1 and insulin-like growth factor-binding protein-1 expression was seen in normal endometrium, supporting previously reported findings.\(^5\) In addition, our observations suggest cycle-specific variations of insulin-like growth factor-1 staining, implying that insulin-like growth factor-1 gene expression may be regulated by the sex steroid milieu of the reproductive tract. These findings are based on the knowledge that the endometrium used as controls was associated with uterine abnormalities (adenomyosis, leiomyomas, benign ovarian cysts) and may not necessarily be normal with respect to the expression of growth factors. Although our sample size was too small to state that there are true cycle-specific variations in insulin-like growth factor-1 protein expression in the endometrium, our results are highly suggestive. These findings suggest a potential role for insulin-like growth factor-1 in secretory differentiation of epithelial cells, growth and decidualization of stroma, and angiogenesis.

Another noteworthy finding in our study is that cyclic variation of insulin-like growth factor-1 immunostaining was also observed in the myometrium. The immunoreactive intensity in the myometrium appeared to be reflective of the level of insulin-like growth factor-1 expressed by the area of most intense endometrial staining. A recent study by Adesanya et al\(^5\) using a primate model supports this observation by showing that insulin-like growth factor-1 messenger RNA levels are upregulated by estrogen and are associated closely with myometrial cell proliferation. These findings and ours suggest that the actions of the insulin-like growth factor system are not specific to the endometrium, but act on uterine tissue as a whole. This is expected because both estrogen and insulin-like growth factor-1 receptors exist in most uterine cell types, and suggests that both receptor pathways are required to regulate uterine physiology.

From our investigation of nine endometrial adenocarcinomas, there appeared to be a progressive decrease in insulin-like growth factor-1 protein expression from well- to poorly differentiated tumors. This finding may suggest that the insulin-like growth factor system plays a role in early carcinogenesis or that insulin-like growth factor-1 expression may simply reflect the stage of differentiation that is lost as neoplastic transformation progresses.

Finally, our study demonstrated increased insulin-like growth factor-1 expression in the endometrium and myometrium of tamoxifen-treated postmenopausal women. This finding was independent of histologic diagnosis of the tissue. Even though many of the tamoxifen-treated specimens displayed proliferative histology, the expression of insulin-like growth factor-1 in the endometrium of tamoxifen patients did not resemble the immunostaining in normal estrogen-dominant proliferative endometrium, but instead, more the pattern found in secretory endometrium. In fact, using image quantitation, tamoxifen was found to significantly increase the staining intensity of insulin-like growth factor-1 to levels greater than that expressed by secretory endometrium. Thus, with tamoxifen administration, there appears to be not only a dysynchrony of insulin-like growth factor-1 regulation in the endometrium, but also a significant upregulation.

An animal study\(^2\) has shown that tamoxifen administration increases uterine volume and insulin-like growth factor-1 expression; however, a review of the literature published during 1966–1997, which was searched in the MEDLINE database using the key words “insulin-like growth factor,” “tamoxifen,” and “endometrium,” was unable to identify any human data that demonstrate the apparent upregulation of insulin-like growth factor-1 expression in the endometrium with tamoxifen therapy. Although not quantitated, myometrial staining of patients receiving tamoxifen resembled the more intense staining found in the myometrium during the secretory phase. Increased insulin-like growth factor-1 expression may be one molecular mechanism that contributes to the increase in uterine volume observed in women receiving tamoxifen therapy by affecting both myometrial and endometrial compartments.

Analysis of insulin-like growth factor-binding protein-1 levels revealed a significant decrease in expression in benign tamoxifen-treated tissue and adenocarcinomas, regardless of tamoxifen administration, compared with controls. Although the exact function of insulin-like growth factor-binding protein-1 is not yet clear, evidence suggests that it may play a role in extracellular transport of insulin-like growth factor-1 and in regulating its bioavailability. Insulin-like growth factor-binding protein-1 is thought to attenuate the bioactivity of insulin-like growth factor-1 by sequestera-
tion. Although the etiology of the reduction of insulin-like growth factor-binding protein-1 expression in benign and malignant tamoxifen-exposed tissues is unclear, one may speculate that lower levels of insulin-like growth factor-binding protein-1 in conjunction with upregulation of insulin-like growth factor-1 expression may further amplify the biologic effects of insulin-like growth factor-1 in the endometrium.

The underlying mechanism by which tamoxifen affects the expression of insulin-like growth factor-1 and insulin-like growth factor-binding protein-1 in the endometrium is not clear. Recent studies\textsuperscript{7\textendash}10 have suggested that tamoxifen can have distinct effects on the expression of genes that do not necessarily mimic the action of estrogen. Three genes have been shown to be specifically upregulated by tamoxifen in breast cancer cells: the quinone reductase gene, which shows so-called reversed pharmacology, being induced by antiestrogens but suppressed by estrogen; transforming growth factor-\(\beta\)-3; and a secreted protein of unknown function.\textsuperscript{7} Tamoxifen also has been shown to specifically downregulate the expression of a number of genes in breast tissue, including transforming growth factor-\(\alpha\), cyclins, c-myc, c-erbB2, and CD36.\textsuperscript{8\textendash}10 On the basis of these studies, one can propose that in the endometrium, tamoxifen may regulate the expression of a subset of genes that are distinct from those controlled by estrogen, and that insulin-like growth factor-1 and insulin-like growth factor binding protein-1 may be one of these genes. Additional studies to investigate the influence of tamoxifen on the endometrial expression of specific genes are warranted.

References


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