

Insulin-like Growth Factor I Gene Expression in the Uterus Is Stimulated by Tamoxifen and Inhibited by the Pure Antiestrogen ICI 182780¹

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Abstract

Estrogen-induced uterine insulin-like growth factor I (IGF-I) expression has been demonstrated to mediate at least in part the uterotrophic action of estradiol. We studied the effects of tamoxifen, a partial antagonist to the estrogen receptor widely used in the treatment of breast cancer, and ICI 182780, a pure antagonist to the estrogen receptor, on uterine weight and uterine IGF-I gene expression in the rat. Tamoxifen increased uterine weight to 125% of control values and doubled uterine IGF-I expression. In contrast, ICI 182780 reduced uterine weight to 60% of control and uterine IGF-I gene expression to 13% of control. These results demonstrate for the first time that uterine IGF-I expression is a molecular marker that correlates with the effects of partial agonists and antagonists to the estrogen receptor on the uterus. Furthermore, the induction of uterine IGF-I expression by tamoxifen provides a molecular mechanism to account for the uterotrophic effects which are commonly seen with tamoxifen therapy and which have been associated with endometrial neoplasia.

Introduction

While the growth response of the uterus to estradiol has been recognized for many years, it is only recently that the role of IGF-I³ as a mediator of estrogen-stimulated uterine proliferation has been appreciated (1, 2). Tamoxifen, a partial antagonist to the estrogen receptor, is widely used clinically because of its demonstrated efficacy in the adjuvant and palliative treatment of breast cancer and is currently being evaluated in large-scale clinical trials as a breast cancer preventative agent (3). A uterotrophic effect of tamoxifen has been described in a variety of experimental systems (reviewed in Ref. 3), but effects of this compound and other antiestrogens on uterine IGF-I physiology have not been described. While tamoxifen therapy is generally well tolerated, postmenopausal women receiving tamoxifen show estrogenic-like changes in the genital tract (4), and use of the drug is occasionally associated with endometrial neoplasia (5–7). Recognition of these issues has had a major impact on the design of tamoxifen prevention trials. The National Surgical Adjuvant Breast Project (NSABP) trial requires that participants undergo annual gynecological examinations, while an Italian tamoxifen prevention trial is open only to women with a prior hysterectomy.

We recently demonstrated that tamoxifen reduces growth hormone output by the pituitary gland (8, 9) and decreases hepatic IGF-I gene expression in experimental animals (10) and serum IGF-I concentration in humans when used in both the adjuvant (11, 12) and preven-

tative (13) settings. Furthermore, we have shown that the inhibitory effect of tamoxifen on hepatic IGF-I expression cannot be entirely accounted for by reduced growth hormone output (10), implying a pituitary-independent inhibitory effect of the drug on IGF-I expression in liver. In view of the clinical and animal model evidence for a uterotrophic effect of tamoxifen, we hypothesized that the effects of tamoxifen on IGF-I expression in the uterus are opposite from the inhibitory effects seen in liver. To investigate this possibility, we used an *in vivo* rat model system to study the effects of tamoxifen and the pure antiestrogen ICI 182780 (14) on uterine weight and uterine IGF-I gene expression.

Materials and Methods

Animals, Drug Administration, and Sample Collection. Pituitary intact and hypox female Sprague-Dawley rats (50 days old; Charles River) were used. We studied control, tamoxifen-treated, and ICI 182780-treated intact animals and similarly treated hypophysectomized and hypophysectomized, growth hormone-replaced animals, for a total of 9 experimental groups. Each experimental group consisted of 4 animals. Tamoxifen and ICI 182780 were administered using a dose and route previously shown to have inhibitory effect on 7,12-dimethylbenz(a)anthracene-induced mammary tumors (15). Specifically, 5 mg tamoxifen (Sigma Chemical Co., St. Louis, MO) or 5 mg ICI 182780 (a generous gift from Dr. A. Wakeling, ICI Pharmaceuticals) were injected s.c. in 0.2 ml peanut oil once daily on 2 consecutive days. Control rats were given s.c. 0.2 ml peanut oil at the same time points. All the animals were sacrificed by CO₂ exposure 7 days after the first day of treatment. Hypophysectomized (Hypox) rats were used 2 weeks after hypophysectomy. For the growth hormone replacement experiments, rats were not only treated with tamoxifen (2 injections for a total of 10 mg on 2 consecutive days) or peanut oil as described above but also were given daily either i.p. saline or human recombinant growth hormone (kindly provided by Genentech, South San Francisco, CA), 100 µg/100 g body weight dissolved in saline i.p. for 7 days, starting on the same day as the tamoxifen or peanut oil. No other pituitary-dependent hormones were administered to hypox animals. All animals were sacrificed by carbon dioxide exposure on day 8. The uteri were excised, immediately frozen in liquid nitrogen, weighed and stored at -70°C for subsequent RNA extraction. Animal experimental protocols were approved by the local animal care committee.

RNA Extraction and Hybridization. Total RNA was isolated from uteri using RNeasy lysis solution and RNeasy spin column (Qiagen, Crawfordsville, IN). For Northern blots, 60 µg of total uterine RNA were used per lane. Separate Northern blots were performed using RNA from each experimental animal. RNA was subjected to electrophoresis through 1.2% agarose gels containing 2.2% formaldehyde. The RNA was transferred onto Zeta-probe membrane (Bio-Rad) in 50 mM NaOH. The blots were hybridized overnight with nick-translated ³²P-labeled rat prepro-IGF-I cDNA (16) (kindly provided by Dr. L. Murphy), dextran sulfate (10%), 1% SDS, herring sperm DNA (500 µg/ml), 0.9 M NaCl, 50 mM Na₂HPO₄·7H₂O, and 5 mM EDTA. The membranes were subjected to three washes at 42°C for 15 min each in solution A (2× SSC-0.1% SDS), solution B (0.5× SSC-0.1% SDS), solution C (0.1× SSC-0.1% SDS), respectively. A final wash was done at 60°C in solution C. The blots were air-dried and subjected to autoradiography for 1 to 3 days with intensifying screen at -80°C. To control for equal loading of wells, we compared total amounts of RNA present in different lanes by rehybridizing the blots with labeled β-actin cDNA (17). Quantitative analysis of gene expression

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³ The abbreviations used are: IGF-I, insulin-like growth factor I; TAM, tamoxifen; hypox, hypophysectomized; cDNA, complementary DNA; SDS, sodium dodecyl sulfate; SSC, saline-sodium citrate; ADU, arbitrary density units; GH, growth hormone treated; ICI, ICI 182780.

was accomplished by scanning autoradiograms densitometrically. For each lane, the sum of the density of bands corresponding to transcripts hybridizing with the IGF-I probe was calculated, and this figure was adjusted for minor differences in RNA loaded (determined as noted above). For each experimental group, we present results by showing a representative Northern blot from one of the four replicates and also show mean densitometry data from the replicates.

Results

The effects of tamoxifen and ICI 182780 on uterine weight in intact rats are illustrated in Fig. 1A (□). While tamoxifen increased weight to approximately 125% of control, ICI 182780 had the opposite effect, with reduction of weight to approximately 60% of control values. Representative Northern blots (Fig. 2A) and the pooled densitometric quantification of IGF-I expression from individual Northern blots from each experimental animal (Fig. 1A, ■) demonstrate the effect of the treatments on uterine IGF-I expression. Following ICI 182780 treatment, uterine IGF-I mRNA levels decreased dramatically compared to control intact rats (Fig. 2A). While ICI 182,780 caused a decrease in IGF-I transcripts, TAM significantly enhanced IGF-I mRNA accumulation (Fig. 2A). Three predominant transcripts with molecular weights of 7.5, 1.4, and 1.0 kilobases were observed. The predominant IGF-I transcripts affected following TAM treatment were those of low molecular weight ranging from 800 to 450 base pairs (Fig. 2A). Densitometric quantitation (the sum of the density of all bands) revealed that tamoxifen increased IGF-I expression from 31.7 ± 3.1 (SD) to 60 ± 7.5 ADU, while ICI 182780 suppressed the expression of this gene to 4.2 ± 1.5 ADU, representing 13% of control, a value even less than that seen in a separate ovariectomized pituitary intact control group, where relative expression was densitometrically quantified as 7.0 ± 3.8 ADU.

Figs. 1B and 2B show the results of similar experiments to examine the effects of tamoxifen or ICI 182780 on uterine weight (Fig. 1B, □), densitometric quantitation of uterine IGF-I transcripts (Fig. 1B, ■), and representative Northern blots (Fig. 2B) of IGF-I gene expression in hypox and hypox, growth hormone-replaced animals. As expected, hypox rats had reduced uterine weight and IGF-I expression compared to controls. Growth hormone treatment of hypox animals resulted in a small increase in uterine weight (from 184 ± 19 to 216 ± 26 mg) and an increase in relative IGF-I expression from 5.4 ± 0.6 to 11.1 ± 1.2 ADU, considerably less than the expression of 31.7 observed in intact animals. Obviously, the growth hormone-replaced hypox ani-

mals remained estrogen deficient, and this result is therefore consistent with the stimulatory role of estrogens in regulating uterine IGF-I expression as previously reported by Murphy and Ghahary (2).

The stimulatory effects of tamoxifen on IGF-I expression and to a lesser extent on uterine weight seen in intact animals were also evident in the hypox and hypox-growth hormone groups. The percentages of increase in uterine weight achieved by tamoxifen were similar in intact (125%) and hypox (131%) animals. In hypox, growth hormone-replaced animals, tamoxifen had a stronger stimulatory effect on IGF-I gene expression than in hypox animals without growth hormone replacement, but this was not associated with a significant increase in tamoxifen-stimulated uterine weight gain. ICI 182780 reduced uterine weight of hypox animals, despite the absence of further reduction of IGF-I gene expression to below hypox levels. In the ICI 182780-treated group of hypox animals, growth hormone replacement was effective at increasing IGF-I gene expression, but the stimulatory effect of growth hormone replacement on weight was attenuated compared to vehicle-treated hypox, growth hormone-replaced animals.

The differences between the following groups with respect to IGF-I gene expression were significant ($P < 0.05$, Mann-Whitney *U* test): control versus tam; control versus ICI; TAM versus ICI; control versus hypox; hypox versus hypox-TAM; hypox versus hypox-GH; hypox-GH versus hypox-GH-TAM; hypox ICI versus hypox-GH-ICI; hypox-TAM versus hypox-GH-TAM.

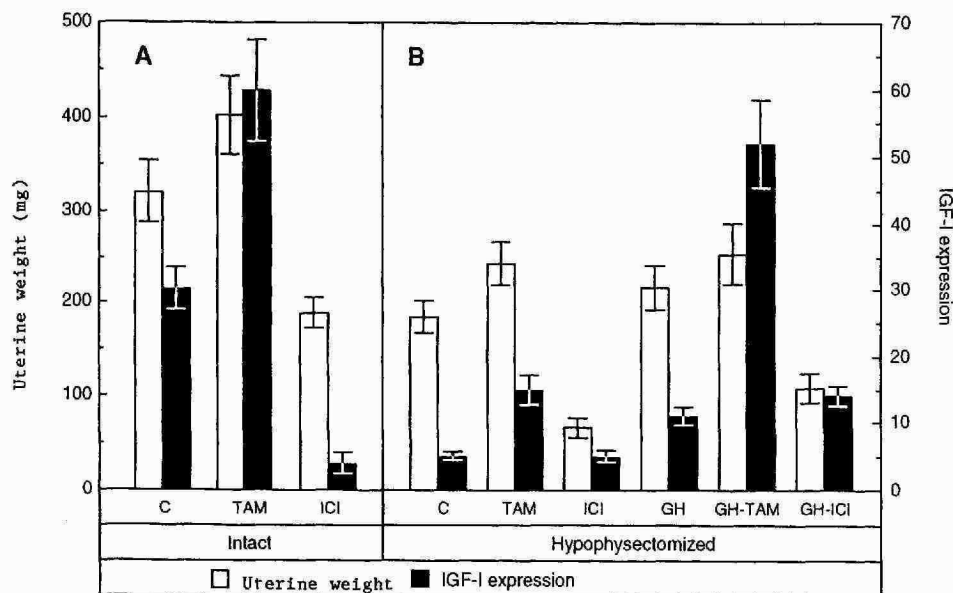
Tamoxifen significantly reduced the serum IGF-I level, as described in our previous report (10). With respect to total body weight, we observed the following. For intact rats: control, 218.6 ± 15.3 g; TAM, 191.4 ± 11.6 g; ICI, 212.6 ± 14.4 g. For hypox rats: hypox, 144 ± 11 g; hypox-GH, 179.8 ± 15.3 g; hypox-GH-TAM, 170.28 ± 12.6 g; hypox-TAM, 131.5 ± 11.7 g; hypox-ICI, 156 ± 18 g; hypox-GH-ICI, 187.6 ± 15.3 g.

Fig. 3 shows the relationship between uterine weight and uterine IGF-I gene expression in all experimental groups. The correlation (Spearman *R*) between weight and IGF-I expression was 0.71 ($P < 0.01$).

Discussion

Estradiol is known to achieve its uterotrophic effect at least in part by stimulating uterine IGF-I expression (2). Our data provide the first evidence to support the hypothesis that the uterotrophic effect of tamoxifen also involves IGF-I as a mediator. The precise mechanism

Fig. 1. (A) Effect of tamoxifen and ICI 182780 on uterine weight and uterine IGF-I expression in intact female rats. (B) Effect of tamoxifen and ICI 182780 on uterine weight and uterine IGF-I expression in hypox and hypox, growth hormone-replaced female rats. Treatments were administered as described in "Materials and Methods." Gene expression was quantified by densitometric scanning of individual Northern blots obtained from individual animals in each experimental group. Mean values \pm SD (bars) are plotted; each experimental group comprised 4 animals. C, control.



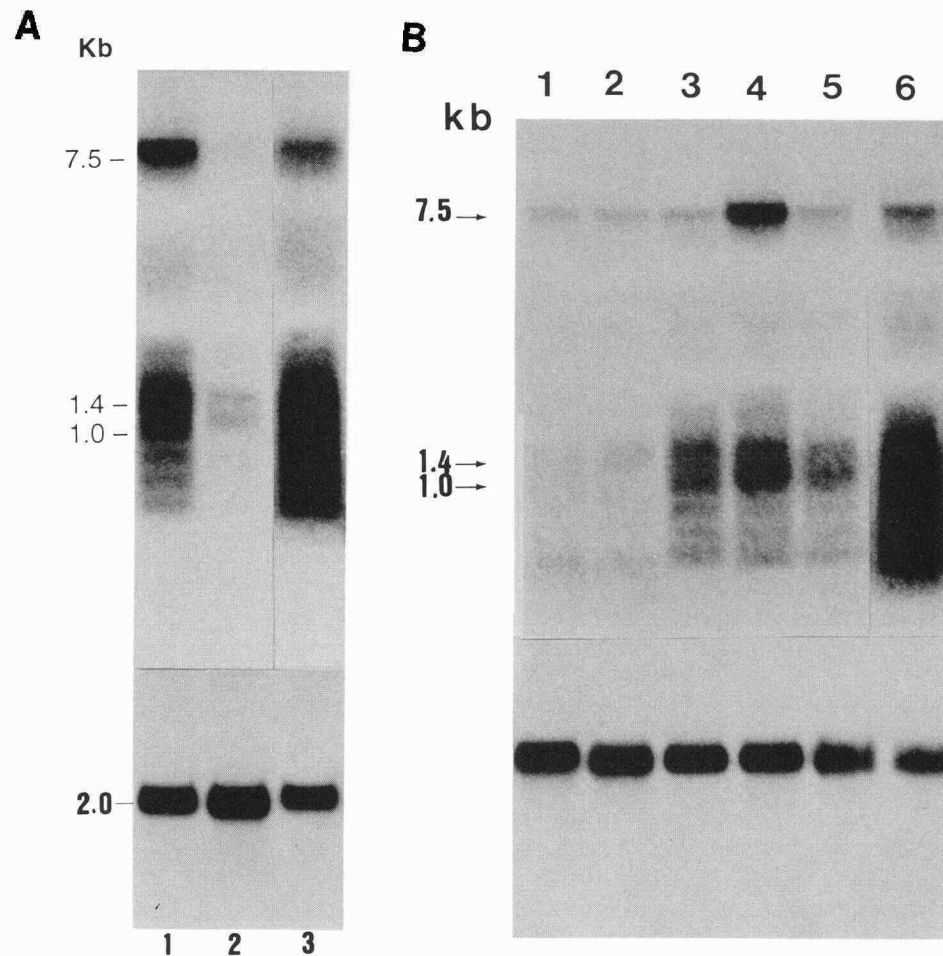


Fig. 2. (A) Representative Northern blots of IGF-I mRNA and control β -actin mRNA [2.0 kilobases (kb)] expression in intact rats treated with vehicle (Lane 1), ICI 182780 (Lane 2), or tamoxifen (Lane 3). (B) Representative Northern blots of IGF-I mRNA and control β -actin mRNA (2.0 kb) expression in hypox and hypox, growth hormone-replaced rats. Lanes 1–3 are from hypox animals that received saline injections, while Lanes 4–6 are from hypox animals that received growth hormone replacement as described in "Materials and Methods." Lanes 1 and 4 are from vehicle-treated animals, Lanes 2 and 5 are from ICI 182780-treated animals, and Lanes 3 and 6 are from tamoxifen-treated animals, as described in "Materials and Methods."

by which tamoxifen increases IGF-I gene expression is not well understood, but IGF-I expression induced by tamoxifen may contribute to the estrogen-like effects of the drug. There is a precedent for this interpretation as Ignar-Trowbridge *et al.* (18) have demonstrated that EGF elicits estrogen-like action in the mouse uterus. The molecular basis for the opposite effects of tamoxifen on IGF-I expression in the uterus as compared to other tissues is under investigation and may be relevant to understanding the tissue-specific differences in agonist versus antagonist effects of the drug.

As both estrogens and antiestrogens have important actions on the pituitary gland (19, 20), we considered the possibility that the effects of tamoxifen and/or ICI 182780 on the uterus were pituitary dependent. The results of our experiments with hypophysectomized animals suggest that growth hormone has a modest stimulatory effect on uterine IGF-I expression but that the effects of tamoxifen and ICI 182780 on the uterus are not mediated via the pituitary. However, the data do demonstrate synergy between tamoxifen and growth hormone with respect to stimulation of uterine IGF-I gene expression, as hypophysectomized animals treated with both growth hormone replacement and tamoxifen showed a level of uterine IGF-I expression greater than the sum of that observed in similar animals receiving growth hormone or tamoxifen individually. This observation is distinct from that observed by Murphy *et al.* in an analogous experiment with estradiol (2) and suggests that the mechanism by which tamoxifen regulates uterine IGF-I expression may not be identical to that of estradiol.

We also demonstrate here for the first time that the reduction of uterine weight following treatment with the complete estrogen an-

tagonist ICI 182780 is associated with inhibition of uterine IGF-I gene expression. The experimental manipulations involving various combinations of growth hormone, tamoxifen, ICI 182780, and hypophysectomy resulted in a greater than 10-fold variation in uterine IGF-I expression. The correlation between uterine weight and IGF-I expression supports the hypothesis that the local expression of this gene is an important determinant of uterine growth. While uterine weight was in general well correlated with uterine IGF-I gene expression, it is noteworthy that in hypox and hypox-growth hormone-replaced animals, ICI 182780 administration suppressed uterine weight significantly, even though there was no significant effect of the drug on IGF-I expression in these animals, which had been rendered estrogen deficient by pituitary ablation. This suggests that ICI 182780 may, in addition to inhibiting IGF-I gene expression in uterus, alter the expression of other genes involved in regulation of proliferation. We therefore are investigating the effects of this compound on IGF-I receptor expression, IGF-I binding protein expression, and growth factors other than IGF-I.

While there is species-to-species variability in the tissue-specific predominance of agonist versus antagonist actions of tamoxifen at the estrogen receptor, the results reported here are relevant clinically, because women treated with tamoxifen frequently exhibit uterine hyperplasia (4) and rarely show neoplasia (5–7). Tamoxifen has been proposed as a treatment for neoplastic conditions of the uterus (21), but results of clinical trials have not been impressive, and there are clinical and laboratory data suggesting that stimulation of endometrial neoplastic growth and leiomyoma growth by tamoxifen are possible (22, 23). It is possible that the effect of tamoxifen on uterine

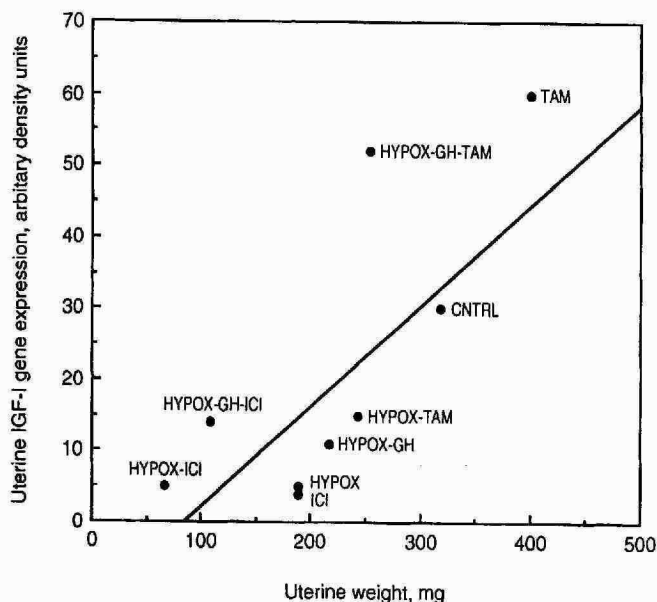


Fig. 3. Scatterplot relating uterine weight and uterine IGF-I gene expression for each experimental condition studied. The abbreviations used in identifying the points are: ICI, ICI-treated; TAM, tamoxifen treated; HYPOX, hypox; GH, growth hormone replaced; CNTRL, control.

IGF-I gene expression which we describe here is related to these adverse effects of the drug. Because IGF-I responsiveness has been reported for endometrial epithelial cells as well as uterine stromal and myometrial cells (2), our results suggest that inhibitory effects of ICI 182780 on IGF-I gene expression may make this agent considerably more useful than tamoxifen in conditions such as endometriosis, leiomyomata, and endometrial carcinoma.

Our data support the view that the use of complete estrogen antagonists instead of tamoxifen would eliminate adverse effects of antiestrogen therapy on the uterus. However, this approach might be problematic because it is likely that the beneficial effects of tamoxifen on serum lipids and bone density (reviewed in Ref. 3) would be lost. We have previously proposed that the coadministration of tamoxifen and a growth hormone-suppressive agent such as somatostatin or a growth hormone-releasing factor antagonist might improve the efficacy of tamoxifen by maximizing the reduction of extrauterine IGF-I expression associated with tamoxifen administration (11, 20). In view of the synergism between growth hormone and tamoxifen in stimulating uterine IGF-I gene expression reported here, it is possible that such a combination would also minimize the adverse effects of tamoxifen on the uterus.

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